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Obesity and adipose tissue expansion in Ossabaw swine

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by

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ABBREVIATIONS

2-NBDG	2-deoxy-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose
AdipoR	adiponectin receptor
AMPK	AMP activated protein kinase
BMI	body mass index
BrDU	bromodeoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation (i.e. CD14)
CCL	chemokine (C-C motif) ligand (i.e. CCL2)
CCR	C-C chemokine receptor (i.e. CCR2)
COX	cyclooxygenase
CXCL	chemokine (C-X-X motif) ligand (i.e. CXCL10
C/EBP	CCAAT/enhancer binding protein
CRP	C-reactive protein
DGAT	diacylglycerol acyltranferase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ERK	extracellular signal-related protein kinase
ERp44	endoplasmic reticulum protein 44
FDR	false discovery rate
FTO	fat mass and obesity associated
G3PDH	glycerol-3-phosphate dehydrogenase
GLUT	glucose transporter
HBSS	Hank's buffered salt solution
HOMA-IR	Homeostasis Model of Assessment - Insulin Resistance
IDO	indoleamine 2,3-dioxygenase
IKKβ	inhibitor of nuclear factor κ B subunit β
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
iNOS	inducible nitric oxide synthase
IRS	insulin receptor substrate
Jak	Janus kinase
JNK	Jun N-terminal kinase
LPS	lipopolysaccharide
M1	classically activated macrophages
M2	alternatively activated macrophages
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MEK	mitogen-activated protein kinase kinase (MAPK/ERK kinase)
MHO	metabolically healthy obese
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	non-esterified fatty acids
NFκB	nuclear factor κ B

NO	nitric oxide
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
PPAR	persoxisome proliferator-activated receptor
PKB	protein kinase B
PUFA	polyunsaturated fatty acids
RNS	reactive nitrogen species
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
SVC	stromal vascular cell
Tlr	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor α receptor
TZD	thiazolidinedione

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ABSTRACT

Obesity is characterized as a state of chronic low-grade inflammation, which originates in adipose tissue from adipocyte dysfunction as a consequence of adipocyte hypertrophy. Although rodent models have been pivotal to understanding the etiology of obesity and its co-morbidities, and the roles of specific genes, distinct differences between rodents and humans underscore the need for alternative comparative models. Swine are an attractive comparative model because of similar physiology and anatomy with humans. Ossabaw swine are of particular interest due to their "thrifty" genotype and predisposition to multiple risk factors for the metabolic syndrome.

Saturated fatty acids have the distinct ability to induce inflammation and insulin resistance via activation of toll-like receptor 4, while n-3 fatty acids generally reduce inflammation and restore insulin sensitivity. Therefore, we first used Ossabaw swine to investigate the effects of high saturated fat and high saturated fat plus n-3 fatty acids on adipose tissue macrophages, inflammation, and insulin resistance. In this study, despite severe obesity, Ossabaw swine did not develop systemic inflammation. Consumption of the high palm oil diet did decrease serum adiponectin, and this decrease was attenuated by the addition of n-3 fatty acids to the diet. Additionally, we determined that the majority of adipose tissue macrophages are of an anti-inflammatory phenotype. High dietary palm oil did increase the percentage of inflammatory macrophages, and this increase was attenuated by the addition of n-3 fatty acids. Finally, the consumption of high palm oil induced insulin resistance, which was attenuated by the addition of n-3 fatty acids. Thus, in Ossabaw swine, morbid obesity induces insulin resistance, but insulin resistance is not linked to inflammation.

Obesity in humans and rodents causes an increase in subcutaneous adipocyte size of ~2 to 3 fold. Ossabaw subcutaneous adipocyte size was increased ~4.3 fold due to the consumption of a diet high in fat. Because of marked adipocyte hypertrophy coupled with the lack of inflammation in obese Ossabaw adipocytes, we sought to determine if Ossabaw adipocytes were protected against saturated fatty acid-induced inflammation. Therefore, we investigated the differences between Ossabaw and Yorkshire stromal vascular cell-derived adipocytes with respect to differentiation and palmitate-induced inflammation and insulin resistance. In this study, we determined that Ossabaw adipocytes express more peroxisome proliferator-activated receptor γ, have more glycerol-3-phosphate dehydrogenase activity, and higher rates of clonal expansion than Yorkshire adipocytes. These finding indicate that Ossabaw adipocytes differentiate to a greater extent than Yorkshire adipocytes. Palmitate decreased insulin-stimulated glucose uptake in both adipocyte genotypes. However, Ossabaw adipocytes had decreased basal and insulin-stimulated glucose uptake compared with Yorkshire adipocytes, although the fold increase was not different with insulin stimulation. With respect to inflammation, palmitate induced nuclear factor κ B activation equally in both adipocyte genotypes, but only Yorkshire adipocytes secreted interleukin 8 in response to palmitate. Consistent with this, there were no differences in serum or adipose tissue IL-8 concentrations between lean and obese Ossabaw swine. Interleukin 8 induces neutrophil chemotaxis into tissues. Neutrophil infiltration into adipose tissue may be important in the early stages of obesity. These data suggest that differences in Ossabaw adipocytes compared with Yorkshire adipocytes may be instrumental in protecting against inflammation during high fat diet-induced obesity.

Finally, we evaluated global gene expression in subcutaneous and visceral adipose tissue of restricted and *ad libitum* fed Ossabaw swine by using an Affymetrix microarray. Microarray analysis revealed no differentially expressed genes between lean and obese Ossabaw swine. This result coincides with no metabolic alterations induced by obesity relating to glucose homeostasis or serum lipids. There were a total of 1494 genes differentially expressed by adipose tissue depot, of which 777 were annotated. DAVID was used to identify functional categories that contained over-represented genes that were differentially expressed by depot. Extracellular space was identified as a gene ontology cellular component category. Two KEGG pathways were identified by DAVID. These were arachidonic acid metabolism and chemokine signaling pathway.

Collectively, we have demonstrated that dietary factors influence the development of metabolic alterations in Ossabaw swine as evidenced by the induction of insulin resistance by high dietary palm oil, the attenuation of insulin resistance by the addition of n-3 fatty acids, and the lack of differentially expressed genes in the adipose tissue of obese Ossabaw swine when consuming a diet without added fat. Additionally, even in the presence of insulin resistance, there was minimal evidence of inflammation beyond the slight increase in the inflammatory $CD16^{-}CD14+$ macrophages in the adipose tissue of Ossabaw swine fed a high dietary palm oil. Palmitate-induced interleukin 8 secretion by Yorkshire adipocytes, but not Ossabaw adipocytes, further supports that Ossabaw swine are resistant to obesity-linked inflammation. The ability of Ossabaw swine to expand adipose tissue may afford protection against the induction of inflammation by diet-induced obesity. Further research into the role of adipose expansion and protection against inflammation is warranted.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

In 2007-2008, it was estimated that 33.9% of the population in the United States is obese (BMI $\geq$ 30) (3), compared with 30.5% in 1999-2000 (4). Obesity and its co-morbidities have placed a significant economic burden on our health care system (20-21; 26). Obesity is linked with a cluster of co-morbidities, that when present, meet the defining criteria for the metabolic syndrome. The metabolic syndrome places individuals at a greater risk for the development of type 2 diabetes (5; 14) and cardiovascular disease (6; 14). Additionally, visceral adiposity and fatty liver associated with obesity pose a greater risk for the development of the metabolic syndrome (9). Because of the increase in prevalence of obesity, there has been increased research effort directed at understanding the etiology of the epidemic and identifying potential interventions.

Adipose tissue, once considered just an energy storage tissue, is now recognized as a fully functional endocrine and immune tissue. This tissue produces hormones, cytokines, and chemokines that modulate metabolic and immunological pathways, both centrally and in peripheral tissues. These molecules are termed adipokines. Chronic positive energy balance induces adipose tissue expansion via adipocyte hypertrophy and/or preadipocyte hyperplasia. Adipocyte hypertrophy and dysregulation leads to an altered adipokine profile that precedes macrophage infiltration (8). Adipose remodeling, or turnover of these "enlarged" adipocytes leads to the recruitment of macrophages, which assist in the necrosis of adipocytes (23). Adipocyte hypertrophy and macrophage infiltration alter adipose tissue production and secretion of adipokines such as tumor necrosis factor (TNF) α, interleukin 6 (IL-6), C-reactive protein (CRP), monocyte chemoattractant protein 1 (MCP-1), IL-8, and adiponectin.

Collectively, obesity-induced dysregulation of these adipokines can induce adipose tissue insulin resistance, leading to ectopic lipid deposition, particularly in the liver (2), muscle (18), and pancreas (22).

Consumption of saturated fat can contribute to adipose tissue inflammation. Saturated fatty acids are a ligand for toll-like receptor 4 (11), the innate immune receptor for lipopolysaccharide, and leads to the activation of nuclear factor κ B (NFκB) and subsequent inflammation. Conversely, n-3 fatty acids are generally considered anti-inflammatory. Eicospentaenoic acid and docosahexaenoic acid are the n-3 fatty acids with biological activity. These n-3 fatty acids can inhibit NFκB (10; 17) and activate peroxisome proliferator-activated receptor γ (1).

Nearly a decade after the link between obesity, inflammation, and insulin resistance was established (7), two laboratories independently discovered that obesity is associated with macrophage accumulation in adipose tissue (27-28). Consistent with this, both body mass and adipocyte size are positively correlated with the number of macrophages present in adipose tissue (27).

Mice have been pivotal to the determination of the role of macrophages in adipose tissue. However, distinct differences between human and murine immune function, particularly regarding the role of CRP as an acute phase protein (24) and nitric oxide production in macrophages (15; 19; 25), limit mice as an immunological model for humans. Swine are increasingly used as a biomedical research model, in large part because of their similar physiology and anatomy with humans (12). Recently, swine have been used to study a number of issues relating to obesity, including cardiovascular disease, steatohepatitis, and insulin signaling. Ossabaw swine are of particular interest, given their “thrifty” genotype due

to genetic isolation (13) and ability to develop all of the risk factors associated with the metabolic syndrome (16).

Despite evidence demonstrating the value of swine as a model of obesity, there has not been an evaluation of adipose tissue inflammation in swine under conditions of diet-induced obesity, particularly regarding macrophage infiltration and induction of inflammation via saturated fatty acids, and the inhibition by n-3 fatty acids. Consequently, there is a clear need to *demonstrate adipose tissue inflammation and macrophage infiltration in Ossabaw swine, and to determine the effects of saturated and n-3 fatty acids on inflammation to further evaluate swine as a comparative model for human obesity.*

Objective and Specific Aims

The *objective* of this study was to determine the extent to which Ossabaw swine develop adipose tissue inflammation and macrophage infiltration, and its effects on insulin sensitivity under conditions of diet-induced obesity. The *central hypothesis* was that diet-induced obesity will cause adipose tissue inflammation concomitant with macrophage infiltration in Ossabaw swine. The *rationale* for our work was to establish a link between adipose tissue inflammation and obesity-linked metabolic consequences in an alternative comparative model for human obesity. Our *specific aims* include i) to determine the effect of saturated and n-3 fatty acids on insulin resistance and macrophage phenotype in Ossabaw swine, ii) to determine the effect of saturated fatty acids on Ossabaw and Yorkshire adipocytes in regards to inflammation and insulin resistance, and iii) identify specific gene categories and pathways in Ossabaw swine adipose tissue that are involved in adipose tissue expansion due to diet-induced obesity.

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CHAPTER 2: LITERATURE REVIEW

Obesity Prevalence

Obesity is an increasing trend in the United States and other developed countries. In 2007-2008, it was estimated that 33.9% of the population in the United States is obese (BMI $\geq$ 30) and 68.3% is overweight (BMI $\geq$ 25) (92). In comparison, the estimates were 30.5% and 64.5%, respectively, in 1999-2000 (93). Obesity and its co-morbidities have placed a significant economic burden on our health care system, and costs are continuing to rise (237; 242; 279). In part, due to public health concern and economic cost, obesity and its co-morbidities have become a large research focus to understand the etiology of the epidemic and to identify potential interventions. However, a myriad of factors, including genotype and environment can contribute to the increasing prevalence of obesity.

Common polymorphisms in the FTO (fat mass and obesity associated) gene and near the melanocortin 4 receptor gene have been associated with the increased risk of obesity. These polymorphisms are associated with increased fat mass and increased risk of obesity (44; 70; 99; 176). Additionally, the presence of multiple risk alleles has an additive effect on the risk of obesity (44). However, the presence of single nucleotide polymorphisms (SNP) cannot completely account for the increased incidence in obesity. For example, the SNP rs1421085 in the FTO gene has an allelic frequency of 0.460 for the risk allele from a US population of European descent, but only a frequency of 0.066 for a United States population of African descent (HapMap). Clearly, more than genetic susceptibility contributes to the increase in obesity prevalence. Thus, genetic susceptibility, in conjunction with urbanization and poor diets has facilitated the increase in obesity prevalence (268).

Obesity is associated with a cluster of co-morbidities that, when present, is defined as the metabolic syndrome. The presence of the metabolic syndrome places individuals at a greater risk for the development of type 2 diabetes (98; 191) and cardiovascular disease (105; 191). The International Diabetes Federation and the American Heart Association/National Heart, Lung, and Blood Institute define the metabolic syndrome as meeting three out of the five following criteria: waist circumference greater than 102 cm for men and 88 cm for women, serum triglycerides greater than 150 mg/dL, HDL cholesterol less than 40 mg/dL for men and 50 mg/dL for women, fasting blood glucose greater than 100 mg/dL, and hypertension with systolic blood pressure greater than 130 mm Hg or diastolic blood pressure greater than 85 mm Hg (7). Additionally, visceral adiposity and fatty liver associated with obesity place individuals at a greater risk for the development of the metabolic syndrome (146).

Obesity, Inflammation, and Insulin Resistance

Adipose Tissue Expansion

Adipose tissue, once considered just an energy storage tissue, is now recognized as a fully functional endocrine and immune organ. This tissue produces a myriad of hormones, cytokines, and chemokines that are capable of modulating metabolic and immunological pathways, both centrally and in peripheral tissues. These molecules, collectively referred to as adipokines, include cytokines, chemokines, adhesion molecules, angiogenic factors, and growth factors that regulate food intake, metabolism, vascularization, reproduction, adipose development, inflammation, and inflammatory cell recruitment (53; 116; 178). Nearly all of the co-morbidities associated with obesity can be linked to chronic inflammation (9; 197; 307).

Chronic positive energy balance induces adipose tissue expansion via adipocyte hypertrophy and/or preadipocyte hyperplasia. Evidence suggests that adipose tissue hypertrophy is genetically limited and varies by depot (69). Adipocyte hypertrophy and dysregulation leads to an altered adipokine profile which precedes macrophage infiltration (131). Death and necrosis of adipocytes, as well as pro-inflammatory adipokine secretion, is positively linked to adipocyte size (54; 129; 250). Adipose remodeling, or turnover of these "enlarged" adipocytes, leads to the recruitment of macrophages, which assist in the necrosis of adipocytes (259). Adipokine-mediated inflammation furthers the recruitment of macrophages and leads to increased inflammation (137; 287). Adipocyte hypertrophy and macrophage infiltration alter adipose tissue production and secretion of adipokines such as tumor necrosis factor (TNF) α, interleukin 6 (IL-6), C-reactive protein (CRP), monocyte chemoattractant protein 1 (MCP-1), IL-8, and adiponectin as well as others.

Adipose tissue inflammation is a potential link between insulin resistance and the eventual development of type 2 diabetes mellitus. It is estimated that 80 % of diabetics are considered overweight and 49 % are obese (204). Thus, there is a strong link between obesity and the development of type 2 diabetes mellitus.

Adipose tissue insulin resistance results in aberrant regulation of the balance between lipogenesis and lipolysis (18). As a consequence, lipolysis is favored and circulating concentrations of free fatty acids are increased. Additionally, postprandial lipid storage is impaired, which further contributes to dyslipidemia (189). As a result, ectopic lipid deposition occurs particularly in the liver (73), muscle (220), and pancreas (246). Ectopic lipid deposition in the liver and muscle is associated with insulin resistance (151; 181), potentially increasing hepatic glucose production and decreasing skeletal muscle glucose

uptake. Furthermore, ectopic lipid deposition in the pancreas can lead to a decrease in β-cell function (246), exacerbating insulin resistance and leading to the development of frank diabetes.

However, some obese individuals do not display the metabolic phenotype often associated with obesity (141; 248). These individuals are termed metabolically healthy but obese (MHO). MHO individuals retain high levels of insulin sensitivity, and have a favorable lipid and inflammatory profile (3; 35; 140; 254). Body composition may be a determining factor in the MHO phenotype. As such, MHO individuals have less visceral (35; 128; 140; 247; 288) and liver (254) fat compared to their metabolically abnormal counterparts. Potentially, the ability to expand adipose tissue may be a fundamental component to the favorable metabolic profile in MHO individuals. In *ob/ob* mice, the induction of adipose tissue expansion attenuates insulin resistance and improves systemic inflammation normally seen in this model, despite morbid obesity (145). This improvement was accompanied by smaller adipocytes and decreased ectopic lipid deposition, suggesting that limits on the ability of adipose tissue to expand cause adipocyte hypertrophy and leads to ectopic lipid deposition that may be causal to the development of insulin resistance in obese individuals.

Insulin Signaling

Insulin is responsible for multiple biological effects, including stimulation of glucose uptake, lipogenesis, protein synthesis, glycogen synthesis, gene transcription, and growth and differentiation. Upon binding of insulin to the insulin receptor, the receptor undergoes auto-phosphorylation of tyrosine residues. Auto-phosphorylation allows the docking and tyrosine phosphorylation of insulin receptor substrate (IRS) proteins (i.e., IRS-1, IRS-2). Tyrosine

phosphorylation of IRS allows the binding of several adaptor proteins including phosphatidylinositol 3-kinase (PI3K). PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate, leading to the down-stream activation of protein kinase B (PKB, i.e., Akt) by phosphorylation of threonine 308 and serine 473, and eventual translocation of GLUT4 to the plasma membrane (as reviewed by Taha and Klip) (266).

Tumor Necrosis Factor α

TNFα is an adipokine that can act both locally and systemically (142). In humans, the production of TNFα is elevated in obesity (142-143; 186), but decreases with weight loss (63; 143). TNFα has two receptors, TNFR1 and TNFR2. TNFR1 is the major receptor in adipose tissue, leading to the activation of nuclear factor κ B (NFκB), and mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated protein kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK) (45). In obesity, elevated TNFα concentrations are directly linked to insulin resistance (123) and cardiovascular disease (249). Additionally, TNFα up-regulates the expression other of pro-inflammatory adipokines (186; 278), and down-regulates the expression of anti-inflammatory adipokines, such as adiponectin (37; 233; 278). At the expense of inflammation and insulin resistance, increased TNFα concentrations during obesity may be viewed as a negative regulator of adiposity and weight gain by increasing lipolysis, inhibiting preadipocyte differentiation, and inducing adipocyte apoptosis (222).

Molecular mechanisms of TNFα-mediated inhibition of insulin signaling involve serine phosphorylation and transcriptional regulation. TNFα inhibits tyrosine

phosphorylation of the insulin receptor and IRS (86), and reduces insulin receptor, IRS-1, and GLUT4 expression in adipocytes (233; 255). One of the best studied inhibitory phosphorylation sites is serine 307/312 (rat/human) of IRS-1. Studies have demonstrated that this residue is phosphorylated by a number of kinases including ERK1/2 (79; 236), JNK (4; 108), IKKβ (107), and mammalian target of rapamycin (mTOR) (213).

Transcriptional inhibition by TNFα is accomplished by inhibitory serine phosphorylation of persoxisome proliferator-activated receptor (PPAR) γ by JNK and ERK (2; 184). The induction of insulin resistance and inhibition of PPARγ by TNFα can be ablated by treatment with thiazolidinediones (TZD) (194; 218; 234). Additionally, TNFα-induced ceramide production is a critical component of insulin resistance (113), leading to inhibition of PKB (269) and decreased CCAAT/enhancer binding protein (C/EBP) α and GLUT4 gene expression (89; 174). Reciprocal positive regulation of both PPARγ and C/EBPα on each other likely leads to a decrease in mRNA abundance. Because GLUT4 is regulated by both PPARγ and C/EBPα (127), the decrease in both is likely causal for the decrease of GLUT4 in TNFα induced insulin resistance. Further transcriptional regulation of insulin resistance by TNFα is evidenced by the induction of suppressor of cytokine signaling (SOCS) 3, which blocks IRS-1 tyrosine phosphorylation and thus inhibits insulin-stimulated glucose uptake (78; 274).

Interleukin-6

Like TNFα, IL-6 production is increased in obesity (100; 142; 277) and decreases with weight loss (16). IL-6 signals through the IL-6 receptor which belongs to the class I family of cytokine receptors and utilizes Janus kinases (Jak) as intracellular signaling

pathways leading to activation of signal transducer and activator of transcription (STAT) 1, STAT3, and MAPK (118-119). IL-6 can act both locally and systemically, and approximately 15-35% of circulating IL-6 is adipose tissue derived in humans (195). In human (276), murine (130), and porcine (302) adipocytes IL-6 induces lipolysis. This effect can be inhibited by PPARγ activation (303). Additionally, IL-6 induces the expression of acute phase proteins (17; 103; 199), a class of proteins secreted predominately by the liver in response to inflammation (i.e TNFα, IL-6).

Literature regarding the ability of IL-6 to induce insulin resistance is much less concise. Similar to TNFα, IL-6 induces the expression of SOCS-3 (85; 152), a key regulatory feature of Jak/STAT cytokine signaling. IL-6 also decreases the expression of the insulin receptor, IRS, GLUT4, PPARγ, and C/EBPα (152; 232), and these effects are reversed with TZD treatment (152). Unlike TNFα, IL-6 does not induce serine 307 phosphorylation of IRS-1, at least in adipocytes (232). However, evidence suggests that IL-6 signaling can induce inhibitory serine phosphorylation of IRS in other tissues (11; 147; 285).

C-Reactive Protein

CRP is an acute phase protein produced in response to inflammation and tissue damage. CRP is produced predominately by the liver (217), but is also produced by adipocytes (43; 210). Increased expression and circulation of CRP in obesity is likely a response to increased adipose tissue production of IL-6 (308). Increased visceral adipose tissue in obesity likely contributes directly to the increase in circulating concentrations of CRP. Indeed, IL-6 is increased in the portal blood of obese individuals, and is correlated to circulating concentrations of CRP (94). Thus, CRP is highly correlated with obesity, insulin resistance, the metabolic syndrome, and the development of type 2 diabetes (48; 64; 97; 101).

CRP concentrations are negatively correlated with the anti-inflammatory, insulin-sensitizing hormone adiponectin (210) and CRP can induce the expression of MCP-1 (215). Additionally, mice ubiquitously over-expressing CRP have more adipose tissue macrophages and increased adipose tissue expression of MCP-1 and TNFα (138). These effects were only seen in high fat diet induced obese mice, not lean controls. The increase in adipose tissue macrophages and increased adipose tissue expression of MCP-1 and TNFα in transgenic CRP mice is likely a result of increased activation of macrophages and other immune cells. In addition to inducing MCP-1 expression (215), CRP activation of leukocytes via FcγII receptor (CD32) (24) increases CCR2 expression on monocytes (115), the receptor for MCP-1, leading to increased chemotaxis into adipose tissue. Moreover, CRP increases macrophage proliferation (68), by increased macrophage colony-stimulating factor production. Thus, CRP is produced in response to inflammation, but has the potential to further propagate the inflammatory condition.

Monocyte Chemoattractant Protein 1

Recruitment of macrophages into adipose tissue in obesity is largely driven by chemokines, such as MCP-1 (CCL2). In obesity, MCP-1 is elevated (36), both in circulation and in adipose tissue. However, increased adipose tissue expression of MCP-1 is not always reflected in the circulation (61). Increased concentrations of circulating MCP-1 is linked to both insulin resistance (267) and cardiovascular disease (185). Knockout of MCP-1 protects against macrophage infiltration into adipose tissue, insulin resistance, and ectopic lipid deposition (137). Conversely, adipose specific over-expression of MCP-1 increases adipose tissue expression of TNFα and IL-6 (136) and macrophage infiltration (136-137), augments insulin resistance (136-137) and leads to increased ectopic lipid deposition (137). Likewise,

knockout of the MCP-1 receptor, CCR2, in mice results in fewer adipose tissue macrophages and decreased inflammation with reduced insulin resistance and hepatic steatosis (137; 286). The importance of MCP-1 in macrophage infiltration is pivotal to the development of insulin resistance due to the contribution to inflammation by infiltrating macrophages. This is further evidenced by the amelioration of insulin resistance in *db/db* mice expressing a mutant dominant-negative MCP-1 (267). Direct contribution of MCP-1 to insulin resistance is less clear. However, pharmaceutical inhibition of MEK attenuates MCP-1 induced insulin resistance (136), at least in myocytes. Potentially, MCP-1 may contribute directly to insulin resistance in other tissues in addition to increasing macrophage infiltration.

Interleukin-8

Like MCP-1, IL-8 is a chemokine that induces leukocyte extravasation, primarily neutrophils, into tissue during an inflammatory response. IL-8 serum concentrations are increased in obesity and are positively correlated to BMI and negatively associated with insulin sensitivity (144). Additionally, IL-8 is correlated to TNFα concentrations in obese subjects (257). IL-8 can be secreted by a number a cell types, and is secreted by adipocytes (122) after challenge with lipopolysaccharide (LPS) and by adipocytes from insulin resistant subjects (232). The ability of adipocytes to secrete IL-8 suggests that adipose tissue has the potential to recruit neutrophils in obesity due to chronic low-grade inflammation. Indeed, neutrophil infiltration in adipose tissue begins after initial stages of high fat feeding in mice (77), and likely precedes macrophage infiltration. Despite the clear association of IL-8 to obesity, the contribution to insulin resistance remains unclear. Evidence of neutrophil infiltration into adipose tissue following initial stages of high fat feeding disappears after prolonged feeding (77). Additionally, circulating IL-8 concentrations paradoxically

increases after weight loss (38), unlike TNFα and IL-6, and therefore is not associated with improvements in insulin sensitivity following weight loss.

Adiponectin

In addition to inflammatory adipokines, adipose tissue also secretes anti-inflammatory adipokines such as adiponectin (i.e., Acrp30, AdipoQ, apM1). Adiponectin is a 30 kDa protein produced predominantly by adipocytes (180; 239) and circulates in three main forms: trimer, hexamer, and high molecular weight (133). The predominate nuclear receptor for transcriptional control of adiponectin is PPARγ (126). Additionally, PPARγ transcriptionally represses the expression of ERp44 (173), which inhibits the secretion of adiponectin from adipocytes (280). Unlike other adipokines, adiponectin is decreased in obesity, insulin resistance, and cardiovascular disease and is negatively correlated with BMI (12; 211). Treatment of human, porcine, and murine macrophages with adiponectin attenuates LPS-induced activation of NFκB and TNFα production (293; 297; 305). Additionally, adiponectin induces the production of the anti-inflammatory cytokine interleukin-10 (IL-10) in porcine macrophages (293). Similar to macrophages, adiponectin attenuates activation of NFκB and expression of TNFα and IL-6 in adipocytes (5).

Perturbed glucose homeostasis caused by insulin resistance can be further impaired by decreased concentrations of the anti-diabetic adipokine, adiponectin. Experimental evidence in adiponectin knockout mice suggests that adiponectin is important in protecting against the development of insulin resistance (150; 202). Adiponectin signals through two receptors, AdipoR1 and AdipoR2, which are differentially expressed on multiple cell types (299). Targeted receptor disruption has demonstrated that AdipoR1 and AdipoR2 signaling activates AMP-activated protein kinase (AMPK) and PPARα (301) , respectively. The

insulin-sensitizing effects of adiponectin mediated by activation of AMPK and PPARα include stimulation of glucose uptake and fatty acid oxidation, and inhibition of lipogenesis and gluconeogenesis (46; 57; 272; 292; 295; 298; 300-301; 306).

Oxidative Stress

Another mechanism of obesity-related insulin resistance includes oxidative stress, which is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress is closely linked with obesity and type 2 diabetes (13; 19; 26). There are a multitude of ROS and RNS that are generated by NADPH oxidase (20), nitric oxide synthase (214), mitochondrial electron transport (312), and cytochrome P450 (309) in response to stimuli such as TNFα (59), endoplasmic reticulum stress (112), insulin (149), and excess nutrients. Oxidative stress induced with hydrogen peroxide *in vitro* has increased activation of JNK and IKKβ, increased serine 307 and 632 phosphorylation of IRS-1, and decreased PI3K activity (25). Additionally, oxidative stress decreases GLUT4 expression in adipocytes (219; 235), thus potentially decreasing insulin stimulated glucose uptake.

Metabolic Endotoxemia

A phenomenon known as metabolic endotoxemia is another contributing factor the development of insulin resistance in obesity. Metabolic endotoxemia is a sub-clinical increase in serum endotoxin, or LPS. LPS initiates a classical immune response via the activation of toll-like receptor 4 (Tlr4), which leads to the production of mediators of inflammation such as TNFα and IL-6. Metabolic endotoxemia is positively correlated with energy intake (8). Consumption of lipids induces postprandial endotoxemia and increases circulating IL-6 (80; 160). Additionally, sub-clinical endotoxemia in humans induces adipose tissue inflammation and transient insulin resistance (190). As such, metabolic

endotoxemia is associated with an increased risk for the development of type 2 diabetes (224).

Obesity and Adipose Tissue Macrophages

Nearly a decade after the link between obesity, inflammation, and insulin resistance was established (123), two laboratories independently discovered that obesity is associated with macrophage accumulation in adipose tissue (287; 296). Both body mass and adipocyte size are positively correlated with the number of macrophages present in adipose tissue (287). In addition to the increase in the number of macrophages, their localization and function change in obese individuals (177). Once within adipose tissue, macrophages recruit more macrophages in a feed forward fashion (206), which increases the inflammatory state and contributes directly to insulin resistance, the metabolic syndrome, and the development of type 2 diabetes.

Human adipose tissue macrophages are characterized via flow cytometry by their expression of CD14, the co-receptor for Tlr4, and CD206, a mannose receptor (310), which distinguishes macrophages from contaminating blood monocytes. Adipose tissue macrophages express high levels of the scavenger receptors CD163 and CD36, do not express the T-cell co-stimulation receptors CD40, CD80, and CD86, and express only low levels of the integrin CD11c (310). One study demonstrated that macrophages expressing CD11c are present in 'crown-like structures' surrounding necrotic adipocytes and are involved in lipid uptake (289), consistent with phagocytic activity. Additionally, adipose tissue macrophages produce high concentrations of IL-1β, IL-6, IL-8, IL-10, TNFα, and MCP-1 (31; 310). Adipose tissue macrophages can also be separated into subpopulations by the expression of CD16, an Fcγ III receptor, with the majority being $CD16^-$ (31). However,

the inflammatory gene expression between $CD16^+$ and $CD16^-$ adipose tissue macrophages differs only by higher expression of MCP-1 and IL-6 in $CD16^-$ macrophages (31). The presence of $CD16^-$ macrophages in adipose tissue may reflect infiltrating monocytes from the circulation (31), as blood $CD16^-$ monocytes express CCR2, the receptor for MCP1 and CD62L (109; 265). However, this has not been definitively proven in regards to adipose tissue macrophages.

Macrophages can be classified into two broad functional subpopulations, which parallels the polarization of naïve CD4+ T-lymphocytes toward either a Th1 or Th2 immune response. The first is classically-activated (M1) macrophages. Activation by interferon γ or LPS in a manner consistent with a Th1 immune response characterize the phenotype of M1 macrophages. These cells produce high amounts of TNFα, and express inducible nitric oxide synthase (iNOS). The second is alternatively-activated (M2) macrophages. Activation by IL-4 or IL-13 in a manner consistent with a Th2 immune response characterizes the phenotype of M2 macrophages. These cells produce high amounts of IL-10, and express arginase instead of iNOS. In adipose tissue, resident macrophages display the M2 phenotype. Adipocyte hypertrophy and dysregulation leads to the monocyte diapedesis to adipose tissue. These infiltrated macrophages are of the M1 phenotype. These two subpopulations of macrophages and their functions in adipose tissue are well reviewed by others (50; 62; 198; 243).

From the initial reports in 2003 by Weisberg *et al.* (287) and Xu *et al.* (296), it has been evident macrophages play a pivotal role in the progression of obesity as a state of chronic low-grade inflammation. Much of the research regarding adipose tissue macrophages has been done in mouse models, including macrophage M1 and M2

polarization. While the M1/M2 phenotype is a useful starting point, human macrophage heterogeneity as well as differences between murine and human macrophages emphasizes the need for alternate immunological models to study macrophage function in relation to obesity.

Macrophage infiltration into adipose tissue is largely driven by MCP-1 and its receptor CCR2. CRP is a acute phase protein that interacts with FcγII receptor (24) and increases CCR2 expression on monocytes (115), as well as induces the expression of MCP-1 (215). However in mice, CRP is not an acute phase protein and is not highly expressed (264), while it is in humans and swine. The lack of CRP functionality in mice does not abrogate the importance of MCP1 and CCR2 in mice models of obesity, as it is evident that they are intricately involved in macrophage infiltration in mice (136-137; 286), but it clearly demonstrates a difference in the etiology of chronic inflammation in obesity between mice and humans.

Probably most notably is the difference between human and murine macrophages is nitric oxide (NO) production via iNOS. Unlike murine macrophages, human macrophages do not produce NO in response to stimuli (192; 231; 270), which is characteristic of M1 macrophages. Consistent with human macrophages, porcine bone marrow-derived macrophages do not produce NO in response to LPS (139). Additionally, porcine bone marrow-derived macrophages up-regulate genes in response to LPS similar to human macrophages, and that are not up-regulated by murine macrophages. These genes include IDO, STAT4, CCL20 as well others. For further review of similarities and differences between pig and human macrophages, please refer to a review by Fairbairn *et al.* (82).

Recently, phenotypical characterization of Yorkshire swine adipose tissue macrophages was performed by flow cytometry (84). Using the mature macrophage marker

CD203a to indentify macrophages, subcutaneous adipose tissue macrophages were predominately CD163+, CD16+, and CD14+ (**Table 1**). It is difficult to make direct comparisons to human adipose tissue macrophages, because the human cells were characterized by being $CD14^+$ in conjunction with $CD206^+$ (310). Swine adipose tissue macrophages are only 80% CD14+ (84). Potentially, the previous studies focusing on human adipose tissue macrophages disregarded populations that are $CD14^-$. Although swine adipose tissue macrophages express higher levels of CD16 (**Table 1**) than humans macrophages do (31), they may still be a valuable resource for investigating the function of macrophages in adipose tissue given the greater differences between mice and human macrophages.

Obesity and Fatty Acids

Multiple studies have demonstrated an association between obesity and increased circulating free fatty acids (121; 223; 227). A contributing factor to elevated circulating free fatty acids is increased adipose tissue lipolysis (1; 120; 153; 230), due in part to the decreased anti-lipolytic effect of insulin. The detrimental effects can be evidenced by the induction of insulin resistance in lean human or rats when infused with free fatty acids (164; 166).

The pathological consequences of increased circulating free fatty acids extend to the activation of Tlr4, an event that further propagates chronic inflammation associated with obesity. LPS recognition by Tlr4 is accomplished through the lipid A moiety (225), which contains saturated fatty acids. Early work by Daniel Hwang's laboratory determined that lauric acid induced cyclooxgenase 2 expression in RAW 264.7 macrophages via the activation of Tlr4 and NFκB (163). In adipocytes, treatment with saturated fatty acids induces inflammation including the activation of JNK and NFκB as well as the production of

IL-6 and TNFα (6; 32; 67; 252; 260). Additionally, *in vivo*, the absence or loss-of-function of Tlr4 protects against high saturated fat induced inflammation and insulin resistance (66; 245; 273). There is also evidence that Tlr2 is involved in saturated fatty acid induced inflammation (65; 67; 76)

Beyond Tlr4, saturated fatty acids can induce inflammation and insulin resistance via ceramide production. Lipolysis can induce inflammation via activation of JNK and p38 MAPK pathways. Inhibition of ceramide synthesis blunts this activation (200) in adipocytes. Moreover, inhibition of fatty acid synthase in adipocytes decreases NFκB activation (6) through the inhibition of ceramide synthesis. Similarly in monocytes, inhibition of ceramide synthesis reduces JNK, ERK, and p38 MAPK activation due to saturated fatty acid treatment (240).

Circulating saturated free fatty acids are positively correlated with circulating IL-6 concentrations (87) and negatively correlated with circulating adiponectin (88). Conversely, n-3 fatty acids are inversely associated with circulating cytokines (90; 134; 193), including CRP, IL-6, and TNFα. The major n-3 fatty acids with biological activity are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are readily available from oily marine fish or can be synthesized *in vivo* from α-linolenic acid. However, it is estimated that only 6% of α-linolenic acid may be converted to EPA or DHA *in vivo* (169). The fate of consumed n-3 fatty acids include incorporation into triglycerides and storage in adipose tissue, incorporation into phospholipids to form part of the cellular membrane, circulation as free fatty acids complexed with albumin, or oxidized for energy.

Several studies have demonstrated the immunosuppressive potential of n-3 fatty acids in multiple cell types including T cells, monocytes, macrophages, dendritic cells, and

adipocytes (6; 124; 205; 311; 313). Observational studies demonstrate a beneficial relationship between n-3 fatty acid intake and obesity, but data from cross-sectional studies are limited (39). In rodents, n-3 fatty acids decrease adipose tissue inflammation, macrophage infiltration, and insulin resistance as well as increase circulating adiponectin (91; 125; 135; 271). In swine, EPA and DHA attenuate LPS induced febrile response and circulating TNFα. (104). Molecular mechanisms of inhibition of inflammation by n-3 fatty acids include alterations in lipid rafts (256; 284), altered eicosanoid production (14; 251), inhibition of NFκB (6; 162; 314), and activation of PPARγ (15; 207).

Swine Models of Obesity

Swine are increasingly being used as a biomedical research model, in large part due to their similar physiology and anatomy to humans (170). In the context of obesity and metabolic syndrome, swine have been used to study cardiovascular disease (22; 27-29; 33; 58; 75; 106; 110; 154; 188; 196; 201; 203; 216; 229; 261; 294), genetic analysis (83; 95-96; 114; 179; 208; 212; 263; 291), drug testing (42; 226; 304; 315), pancreas function (102; 155; 157), dietary components (34; 221; 228-229; 238), steatohepatitis (21; 165), renal function (47; 168; 171), insulin signaling (161), and exercise training (58; 75).

Commercial swine fed diets high in fat and cholesterol have been used to study diabetes accelerated atherosclerosis (110; 201; 261). These swine developed hypercholesterolemia, but had normal circulating concentrations of glucose and triglycerides. Development of diabetes by treatment with streptozotocin was required to induce hyperglycemia. Additionally, streptozotocin exacerbated the progression of atherosclerosis in this model. Consumption of high fat diets by commercial swine increases abdominal fat, weight gain, and results in hypertension and hypercholesterolemia (106). However, it does

not significantly affect insulin sensitivity or increase circulating concentrations of CRP (106). As mentioned previously, increased circulating free fatty acids and sub-clinical endotoxemia are physiological responses associated with obesity. In commercial swine, experimental endotoxemia and increased circulating concentrations of free fatty acids did not affect glucose homeostasis, but did decrease circulating growth hormone concentrations (40), which may increase adipose tissue mass and decrease lean mass.

Commercial swine have also been used to study the effect of consumption of different fats on body composition and metabolic parameters. Saturated fats (lard), mono-unsaturated fats (olive oil), and sucrose consumption increased whole-body adiposity, but only the mono-unsaturated fat increased visceral adiposity (238). Consumption of either saturated or mono-unsaturated fat, but not sucrose, decreased insulin sensitivity (238). Another study evaluated the effect of the consumption of saturated fats (lard) and polyunsaturated fats (canola oil) on metabolic markers and inflammation. Neither fat source altered circulating IL-6 or CRP concentrations, increased adipose tissue macrophages, or altered insulin sensitivity; although the saturated fat induced hypercholesterolemia (34). Differences in the ability of commercial swine to develop dietary induced changes in insulin sensitivity may be affected by the age that dietary treatment was initiated. Sabin *et al.* (238) began treatment at one week of age, Galili *et al.* (106) began treatment at 3 months of age, and Braucher (34) began treatment at 18 weeks of age. Increased age of initial dietary treatments may reduce the negative effects of dietary fat on insulin sensitivity.

Although commercial swine have been used in obesity-related research, common genetic selection for lean growth limits the potential of this model. Ossabaw swine, however, are a promising model for obesity research, and have already been extensively used

in cardiovascular disease and steatohepatitis research (22; 27-29; 74-75; 154; 165; 203; 216). Ossabaw swine were isolated for ~500 years on Ossabaw Island, just off the coast of Georgia, USA (187). The introduction of new genetics has been limited, but includes one Hampshire boar in the mid 1970s (187). This genetic isolation allowed for the development of a "thrifty genotype" to survive the famine and feast environment. Because of this unique adaptation, Ossabaw swine can store more fat on a kg body weight basis than any other non-domesticated mammal (258).

Ossabaw swine are smaller, slower growing, and have decreased muscle mass compared to domestic swine (41; 60; 182). Increased fat storage and decreased muscle mass in Ossabaw swine may be due to decreased growth hormone secretion in response to insulin (283), but not hyperphagia (281). Ossabaw swine have impaired glucose tolerance (282) and hypertriglyceridemia and hypercholesterolemia (81) as well as a higher capacity for gluconeogenesis, glycolysis, and lipogenesis than lean swine (183).

The aforementioned information regarding Ossabaw swine was collected in the 1970s. Recently, growing concern about the obesity and diabetes epidemic sparked new interest in Ossabaw swine and a breeding colony was established by Michael Sturek (262). Phenotypically, Ossabaw swine display many of the same characteristics today as they did in the 1970's. Upon development of obesity, they develop hypercholesterolemia (23; 27; 29; 56; 71; 74; 148; 159; 165; 168; 175; 203; 216), hypertriglyceridemia (27; 29; 71; 74; 148; 165; 216), impaired glucose tolerance (74; 159; 203), hyperglycemia (27; 29; 165; 216), and hyperinsulinemia (27; 29; 71; 165). It is important to note that in addition to high fat content, some of the diets contained supra-physiologic amounts of cholesterol (23; 27; 29; 33; 56; 74; 148; 159; 165; 168; 175; 203; 216), which may augment the dyslipidemia in this model.

Only one study showed a decrease in serum adiponectin, marked hyperlepinemia, and systemic inflammation upon prolonged consumption of a high fat diet (165).

Ossabaw swine contain a mutation in the γ3 subunit of AMPK (172). This mutation (V224I) is a Val^{199}→Ile missense mutation that causes low muscle glycogen and increased intramuscular fat (10; 55). This mutation is consistent with the increase in intramuscular lipid seen in obese Ossabaw swine (56). It has been hypothesized by Shen et al. that this mutation may be causal in the development of insulin resistance in Ossabaw swine (244). However, to this date this has not been investigated.

Another common swine model is the Yucatan minipig. Yucatan swine originate from the Yucatan Peninsula of Mexico. When Yucatan swine are fed a high fat high, cholesterol diet, they develop hypercholesterolemia (72; 111; 196; 209; 290), but do not exhibit decreased insulin sensitivity in response to an insulin challenge (209). Yucatan swine do not readily develop hyperglycemia upon consumption of a western diet (30; 72; 196; 209; 241; 290), but do have a decreased glucose infusion rate during the hyperinsulinemic-euglycemic clamp (241). Additionally, Yucatan swine fed a high fat high cholesterol diet have higher liver content of cholesterol, cholesteryl esters, and triglycerides (72), as well as increased visceral adiposity (290). When fed a western diet, Yucatan swine develop hyperleptinemia (241; 290) and have increased adipose tissue expression of leptin and IL-6 (241). Whereas previous studies in Yucatan swine showed only hypercholesterolemia and minimal effect on glucose homeostasis; more recently Yucatan swine have been shown to develop increased circulating concentrations of triglycerides and NEFA, as well as hypercholesterolemia after long-term consumption of a high fat diet (161). In addition, these swine develop hyperglycemia, hyperinsulinemia, and impaired myocardial insulin signaling (161).

Differences between these studies may be attributable to varying genetics between breeding herds.

Göttingen minipigs are another swine model that are used to study obesity and its co-morbidities. Göttingen swine were developed in the 1960s in Göttingen, Germany by cross breeding between Vietnamese swine, Hormel swine, and the German improved Landrace (156). Gottingen swine fed a high fat diet have increased body fat (132; 158; 275), plasma cholesterol (158) and triglycerides (132). Göttingen swine display insulin resistance after 3-5 months of high fat feeding (158; 275), as well as hyperinsulinemia in response to an intravenous glucose tolerance test after as little as 5 weeks of high fat feeding (132). However, impaired fasting glucose was only seen in one study (158). Additionally, there is a sexual dimorphism in the susceptibility of Göttingen swine to negative metabolic effects during diet-induced obesity. Female Göttingen swine become more obese, have a greater degree of insulin resistance, and a more atherogenic plasma profile (i.e. cholesterol, triglycerides) than their male counterparts (52). Higher concentrations of both testosterone and estradiol (52) in male Göttingen swine may reduce their risk for obesity-associated co-morbidities (51).

In addition to Ossabaw, Yucatan, Göttingen, and commercial swine, there are several Chinese swine that have been used in obesity research. These include Guizhou, Ningxiang, Bama, Wuzhishan, and Nongda swine. When fed a high fat, high sucrose diet, Guizhou swine have increased body weight gain and increased fat deposition (294; 304). These swine develop dyslipidemia (294; 304) , hyperglycemia (294), and display insulin resistance as evidenced by both an insulin challenge and an oral glucose tolerance test (294; 304). Additionally, ectopic lipid deposition is evident in the liver, pancreas, and muscle after long

term dietary treatment (294; 304). Moreover, Guizhou swine have increased circulating concentrations of TNFα after long-term dietary treatment (294). The genetically obese Chinese Ningxiang swine may have potential as an obesity model. Compared to lean commercial swine Ningxiang have higher circulating concentrations of insulin and cholesterol, as well as greater adipose tissue and less lean mass percentage (117). In a direct comparison of Bama, Wuzhishan, and Nongda swine, only the Bama and Wuzhishan swine developed hyperglycemia in response to a high fat and high sucrose diet (49). Additionally, Bama swine fed a high fat, sucrose, and cholesterol diet developed hyperglycemia, hyperinsulinemia, hypercholesterolemia, and also had decreased circulating concentrations of IGF-1 (167).

While rodent models have been a pillar to obesity research, they do not consistently develop three or more of the risk factors of the metabolic syndrome (253). The ability of Ossabaw and Yucatan swine to develop features of metabolic syndrome has been compared by Neeb *et al.* (203). Yucatan swine only develop hypercholesterolemia, while Ossabaw swine develop obesity, insulin resistance, glucose intolerance, hypertriglyceridemia, hypercholesterolemia, and hypertension (203). Additionally, as reviewed above, Ossabaw swine consistently develop more risk factors for the metabolic syndrome than other swine as breeds. While Ossabaw swine do develop obesity and insulin resistance, adipose tissue inflammation has yet to be evaluated in this breed, or any other breed. Only one study demonstrated increased systemic inflammation in Ossabaw swine (165), but did not evaluate adipose tissue inflammation. Chronic low-grade inflammation is a hallmark of obesity and thus, needs to be evaluated in Ossabaw swine to further the development of the model.

Summary

The prevalence of obesity and its co-morbidities continues to rise in the United States and other developed countries. Excess caloric intake causes adipose tissue expansion through adipocyte hypertrophy and hyperplasia. Adipocyte hypertrophy leads to adipose tissue dysfunction causing increased production of inflammatory adipokines (i.e. IL-6, TNFα, MCP-1) and a decrease in anti-inflammatory adipokines (i.e. adiponectin). These events lead to macrophage infiltration into adipose tissue, propagating inflammation and creating a chronic state of low-grade inflammation that is often associated with obesity. The increased production of inflammatory adipokines by adipocytes and macrophages, as well as oxidative stress induces adipose tissue insulin resistance via the activation of NFκB and MAPK pathways. Free fatty acids released from adipocytes due to insulin resistance, as well as from the breakdown of necrotic adipocytes by macrophages causes ectopic lipid deposition, particularly in muscle, liver, and pancreas. Ectopic lipid deposition, in conjunction with increased systemic inflammation from adipokines, can lead to insulin resistance in the liver and muscle and decrease pancreatic insulin secretion. These events can lead to the eventual development of type 2 diabetes mellitus.

The susceptibility to obesity is influenced by multiple factors including genotype, environment, and poor diet. Dietary factors, such as fatty acids, have potential implications in obesity. Saturated fatty acids are linked to the inflammatory profile in obese individuals. Saturated fatty acids can activate Tlr4, activating NFκB and MAPK pathways and thus, increasing inflammation. Additionally, consumption of saturated fatty acids can contribute to metabolic endotoxemia. Conversely, the n-3 fatty acids EPA and DHA are anti-inflammatory in nature and are negatively correlated to the inflammatory profile in obese

individuals. EPA and DHA increase PPARγ activation and adiponectin production, and decrease NFκB activation.

Largely because of comparative physiology and anatomy, the use of swine for research involving obesity and its co-morbidities has increased. Ossabaw swine in particular become obese when adequate food is available and develop multiple risk factors of the metabolic syndrome including insulin resistance. However, adipose tissue inflammation has not been investigated in this model. Macrophage infiltration and function in adipose tissue is of particular interest due to immunological discrepancies between mice and human, especially regarding CRP function and NO production by macrophages. Swine are a more relevant immune model for humans, but needs further evaluation. Based upon the literature outlined above, I sought to characterize adipose tissue inflammation in Ossabaw swine in response to saturated and n-3 fatty acids and to determine specific genes involved in adipose tissue expansion by diet-induced obesity to further validate Ossabaw swine as a model for human obesity and obesity-linked inflammation.

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Tables

Table 1. *Swine subcutaneous adipose tissue macrophage phenotype*[a]**.**

Marker	Mean (%)	SEM
CD203a+	26.94	2.29
$CD163^{+}$	69.82	1.12
$CD14^{+}CD16^{+}$	67.64	2.60
$CD14^{+}CD16^{-}$	20.17	2.37
$CD14^{-}CD16^{+}$	7.52	0.25
$CD14^{-}CD16^{-}$	4.66	0.38
$CD163^{-}$	30.18	1.12
$CD14^{+}CD16^{+}$	41.15	1.41
$CD14^{+}CD16^{-}$	21.99	0.81
$CD14^{-}CD16^{+}$	12.32	0.41
$CD14^{-}CD16^{-}$	24.54	1.15

[a]Adapted from Faris *et al*. (84).

CHAPTER 3: INFLAMMATION AND RESPONSE TO N-3 FATTY ACIDS IN A PORCINE OBESITY MODEL

Accepted by *Comparative Medicine*

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Abstract

Fatty acids have distinct cellular effects relating to inflammation and insulin sensitivity. Dietary saturated fat activates toll-like receptor-4 which can lead to chronic inflammation, insulin resistance, and adipose tissue macrophage infiltration. Conversely, n-3 fatty acids are generally anti-inflammatory and promote insulin sensitivity, in part via through peroxisome proliferator-activated receptor-γ. Because Ossabaw swine are an attractive biomedical model, we fed Ossabaw pigs either a low fat control (LFC), high fat palm oil (HFP), or high fat palm oil with n-3 fatty acids (HFPn3) diet for 30 weeks to investigate the effect of saturated fats and n-3 fatty acids on obesity-linked inflammatory markers. There was no diet-related induction of the inflammatory markers C-reactive protein, tumor necrosis factor-α, interleukin-6 or interleukin-12. The increase in inflammatory adipose tissue $CD16^{-}CD14^{+}$ macrophages by high palm oil was attenuated by n-3 fatty acids. Both high fat diets induced hyperglycemia, without hyperinsulinemia. Insulin sensitivity was decreased in the HFP group, but not the HFPn3 group during an insulin challenge. This effect was not mediated by decreased phosphorylation of PKB. Thus, in a porcine model of obesity, n-3 fatty acids partially attenuate insulin sensitivity, but only marginally change inflammatory status and macrophage phenotype in adipose tissue.

Introduction

Obesity is accompanied by chronic inflammation in adipose tissue, increased circulating concentrations of tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and C-reactive protein (CRP), and decreased concentrations of adiponectin (2). This chronic inflammation links obesity and the development of insulin resistance (39). Dietary saturated fatty acids promote obesity in part through the induction of inflammation via activation of toll-like receptor-4 (i.e., tlr4, the innate immune receptor for lipopolysaccharide) (28). Absence of functional tlr4 in mice reduces circulating proinflammatory cytokine concentrations and decreases macrophage infiltration into adipose tissue in high fat diet-induced obesity (8; 28; 32). Furthermore, in 3T3 L1 mouse adipocytes, palmitate activates nuclear factor κB (NFκB), protein kinase C, and mitogen-activated protein kinase, all of which increase inflammatory cytokine production (1).

For individuals consuming a diet high in saturated fat, a major determinant of health is the n-6:n-3 (fatty acids, omega-6:omega-3) (5-6). Unlike saturated fatty acids, the n-3 polyunsaturated fatty acids (PUFA) eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) exert predominantly anti-inflammatory effects, as is evident in that DHA antagonizes NFκB activation by palmitate in 3T3 L1 adipocytes (1). In mice, EPA prevents or reverses hyperinsulinemia, hyperglycemia, and increased circulating monocyte chemotatic protein-1 (MCP-1) (16), and also decreases infiltration of adipose tissue with macrophages (30). Moreover, n-3 PUFA alleviate the decline in serum adiponectin associated with obesity (12; 15; 30), and EPA decreases serum CRP in diabetic patients (26).

Physiological differences between rodents and humans underscore the need for comparative models in biomedical research, and the pig is emerging rapidly as an attractive

model for energy metabolism and obesity. Like humans, pigs are natural omnivores, rely on apo B-100 to shuttle cholesterol in the LDL fraction, and have minimal brown fat retention postnatally. Furthermore, adipose depots in pigs are of sufficient size that multiple assays can be carried out on adipocytes or stromal vascular cells without pooling across depots or animals. Although Ossabaw swine have been used as a model for metabolic syndrome, cardiovascular disease, coronary artery disease, and steatohepatisis (11; 20; 24), very little is known about adipose inflammation in this model. Consequently, we sought to characterize obesity-linked inflammatory markers in the adipose tissue of this novel model, and to test the hypothesis that adding n-3 PUFA to a diet high in saturated fat will attenuate chronic inflammation, protect against diet-induced insulin resistance, and alter phenotypical changes in adipose tissue macrophages.

Methods

Animals and Diets. All animal procedures were conducted at Iowa State University and were approved by the Iowa State University Institutional Animal Care and Use Committee. Two groups (27 total) of mature male and female Ossabaw swine (*Sus scrofa*) (initial body weight 47.4 ± 0.3 kg) were housed in individual pens in a temperature controlled environment with a 12 hour light cycle. Swine were obtained from the Comparative Medicine Program at the Indiana University School of Medicine and Purdue University from a herd that tested negative for *Brucella* spp. and psuedorabies, and showed no clinical evidence of communicable disease upon arrival or during the study. Animals were assigned to one of three dietary treatments: a low fat control diet (LFC, n=9; 3 male and 6 female), a high fat palm oil diet (HFP, n=9; 2 male and 7 female), and a high fat palm oil diet with n-3 supplementation (HFPn3, n=9; 2 male and 7 female) (**Table 2**). Three pigs in

the HFPn3 dietary treatment died during the study. Autopsy results indicated that the pigs did not have any obvious signs of disease. It was noted that severe congestion in the lungs would support a heart condition that was acute and fatal. However, the exact cause of death was unknown. The HFP and HFPn3 diets were fed *ad libitum* and the LFC diet was restricted to maintain a mature lean body weight. The "healthy" control diet (**Table 2**) was designed to limit caloric intake and provide adequate n3 fatty acids through modest inclusions of fish meal, canola oil, and n3 fatty acid supplement (Gromega^{TM} 365). Both high fat diets were high in saturated fatty acids and formulated to be isocaloric. Canola oil and the n3 fatty acid supplement was used to reduce the n6:n3 to be comparable with the control diet. Body weight and feed intake were measured weekly. To confirm the fatty acid profile of each diet, lipids were extracted by the method of Lepage and Roy (21). Fatty acid methyl esters were analyzed by gas chromatography (model 6890; Hewlett-Packard, Palo Alto, CA) fitted with an Omegawax 320 (30 m x 0.32 mm internal diameter, 0.25 μm) capillary column (Sigma-Aldrich, St. Louis, MO). The injector and detector temperatures were 250°C, and the oven temperature was 200°C.

Insulin Challenge and Tissue Collection. After 30 weeks of dietary treatment, animals were fasted for 12 hours overnight and were anesthetized with telazol:ketamine:xylazine (1:1:1). Thereafter, blood pressure and mean arterial pressure were taken with an oxillometric blood pressure monitor (Cardell, CAS Medical Systems, Inc., Branford, CT) with a 9 cm cuff on the metatarsus. Each measurement was taken three times and averaged. Blood was collected via jugular venipuncture and then animals were challenged with 0.25 IU/kg of porcine insulin (MP Biomedicals, Solon, CA) intravenously as adapted from Xi et al. (37). Glucose measurements were taken from blood obtained by ear

pricks at 0, 5, 10, and 15 minutes post insulin challenge using a glucometer (LifeScan, Milpitas, CA). Pigs were then killed by exsanguination. Subcutaneous (SQ) adipose tissue from back fat above the 10^{th} rib, visceral adipose tissue (VIS) from the ventral intra-abdominal cavity, omental adipose tissue (OM), liver, and longissimus dorsi (LD) from above the 10^{th} rib were collected. Liver and LD were snap frozen in liquid nitrogen. Adipose tissue was divided two ways: snap frozen in liquid nitrogen or stored in ice cold PBS with 0.2% BSA for transport to the laboratory and the isolation of stromal vascular cells (SVC).

SVC were isolated from adipose tissue as follows: adipose tissue was minced finely with a razor blade and digested in Hank's Buffered Salt Solution (HBSS) with 20 mM HEPES (pH 7.4), 3% BSA, and 2 mg/mL Collagenase Type I (Worthington-Biochemical Corporation, Lakewood, NJ) for 45 minutes, 125 rpm, at 37°C. Samples were then centrifuged for 5 min at 1000xg and the supernatant was discarded. The pellet was dissolved in 10 mL of RBC lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 5 min to lyse red blood cells, and then 10 mL of PBS was added and samples were passed through a 100 μm filter. The filtrate was centrifuged for 5 min at 1000xg and the pellet was resuspended in HBSS with 20 mM HEPES (pH 7.4).

Flow Cytometry. Up to 1 x 10^6 SVC were washed in 2 mL of fluorescence-activated cell sorting buffer (FACS, 0.1% sodium azide, 0.1% BSA in PBS). Cells were treated with 10 μL of pig serum for 15 min at 4ºC to prevent nonspecific binding. Cells were then incubated with 10 μL of anti-CD203a IgG_1 (1:20) (Mybiosource, LLC, San Diego, CA) or isotype control (mouse IgG_1, κ, 1:20) (eBioscience, Inc., San Diego, CA) for 15 minutes at 4ºC and then washed in 2 mL of FACS buffer. Cells were next incubated in 10 μL of goat-

anti mouse IgG_1-FITC (1:50) (SouthernBiotech, Birmingham, AL) for 15 min at 4ºC and then washed in 2 mL FACS buffer. Cells were incubated in 10 μL of biotinylated anti-CD16 (1:50) (BD Pharmingen, San Diego, CA) or isotype control (biotinylated mouse IgG_1, κ, 1:50) (BD Pharmingen) for 15 min at 4ºC and then washed in 2 mL of FACS buffer. Cells were then incubated in 10 μL of Streptavidin-Peridinin Chlorophyll-a Protein-Cy5.5 (1:50) (BD Pharmingen) and 10 μL of anti-CD14-PE (1:20) (Antigenix America, Huntington Station, NY) or isotype control (PE mouse IgG_{2b}, κ, 1:20) (BD Pharmingen) for 15 minutes at 4ºC and then washed in 2 mL of FACS buffer. Cells were then fixed in 200 μL of 1% paraformaldehyde and stored at 4ºC until analysis. Analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA), and data was analyzed using FlowJo software V8.5.2 (Tree Star, Inc., Ashland, OR).

Serum and Tissue Analyses. Cholesterol, triglycerides, and non-esterified fatty acids (NEFA) were analyzed using Chol Slides (Ortho Clinical Diagnostics, Rochester, NY), Trig Slides (Ortho Clinical Diagnostics), and NEFA-HR (Wako Diagnostics, Richmond, VA), respectively, on a Vitros 5.1 Chemistry Analyzer (Ortho Clinical Diagnostics). Serum insulin and CRP concentrations were measured by EIA and ELISA, respectively (ALPCO, Salem, NH). Serum TNFα, IL-6, IL-10 and IL-12p40 concentrations were measured by ELISA (R & D Systems, Minneapolis, MN). The HOMA-IR was calculated using fasting serum insulin (μU/mL) x fasting blood glucose (mg/dL) divided by 405 (23). Plasma homocysteine concentrations were determined by HPLC as described by Williams et al. (36). Total liver and LD lipid content was determined by the method of Folch et al. (13).

Whole frozen tissue samples were pulverized and homogenized in ice cold buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM NaF, 5 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10% glycerol, and 1% Triton X-100). Homogenates were mixed by shaking for 45 min at 4°C and then centrifuged at 10,000xg for 20 min at 4°C. The supernatants were collected and the protein concentration was determined using BCA reagent (Pierce, Rockford, IL).

Samples were added to a reducing loading buffer with a final concentration of 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromophenol blue and heated for 10 min at 100°C. Equal amounts of protein were separated by SDS-PAGE (10% resolving gel), transferred to a nitrocellulose membrane and probed with either an anti-adiponectin rabbit polyclonal antibody (1:750) (Alpha Diagnostics, San Antonio, TX), anti-Ser_{473} PKB rabbit polyclonal antibody (1:2000) (Cell Signaling Technology, Danvers, MA), anti-PKB (1:1000) (Cell Signaling Technology), or anti-AMPKα (1:1000) (Cell Signaling Technology) overnight at 4°C. Both the total and phospho-specific PKB antibodies detect all isoforms. The porcine adiponectin antibody was generated against the following two peptides: DQYQDKNVDQASGS and TEKPGALLPVPKGAC. Anti-adiponectin was purified from immunized rabbit serum by affinity chromatography. Membranes were then probed with a goat anti-rabbit IgG horseradish peroxidase (1:10,000) (Pierce) for 1 hour at room temperature. Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce) and imaged with FOTO/Analyst Luminary FX system (Fotodyne Incorporated, Hartland, WI). Densitometry was performed using TotalLab TL100 software (TotalLab Ltd, Newcastle, UK). Serum (2 μL) was diluted in

reducing loading buffer, heat denatured, and separated by SDS-PAGE as described above for estimation of serum adiponectin concentrations. Phosphorylation of AMPKα at Thr_{172} was measured by ELISA (Cell Signaling Technology).

Statistical Analysis. All statistical procedures outlined were performed using SAS 9.2 (SAS, Cary, NC). Residuals were analyzed to detect outliers and assess normality using the PROC Univariate procedure. Right-skewed data were log-transformed for further analysis. ANOVA was performed using the PROC GLM procedure. Main effects included diet and tissue where appropriate. Fixed effects include sex, group, and termination date. Fixed effects were used in the model when $p \leq 0.15$ for the fixed effect or interaction with main effect(s). Blood glucose data from the insulin challenge were analyzed using an autoregression order of one repeated measures. For flow cytometry data analysis was weighted on live cell number. Additionally, $\%CD203a^+$ was used as a covariate for analysis of CD14 and CD16 data. Data presented are least-squares means ± standard error.

Results

Dietary fatty acid profiles are presented in **Table 3**. Only the LFC and HFPn3 diet contained measurable EPA and DHA while all three diets contained α-linolenic acid. The addition of n-3 PUFA to the HFPn3 diet decreased the n-6:n-3 from 47.6 in the HFP diet to 3.9 in the HFPn3 diet. The average calculated n-3 PUFA consumption was 0.40, 0, and 7.6 g/d for EPA, and 0.36, 0, and 6.7 g/d for DHA for the LFC, HFP, and HFPn3 groups, respectively. To maintain lean mature body weight, caloric intake was limited in the LFC group to an average of 1778 kcal/d compared to ~9800 kcal/day for the two high fat diets. Final body weights for the two high fat groups did not differ (**Table 4**).

To assess the impact of the high fat diet and n-3 supplementation on systemic metabolic disturbances, insulin, blood glucose, cholesterol, triglycerides, homocysteine, and blood pressure were measured (**Table 4**). There was no difference detected among the LFC, HFP, and HFPn3 groups in fasting serum insulin, cholesterol, triglyceride, or homocysteine concentrations. In contrast, there was a dietary effect with serum NEFA; the HFPn3 group had higher NEFA concentrations than the LFC group, whereas the HFP group was not different from either the LFC or the HFPn3 group. Additionally, consumption of either high fat diet increased systolic blood pressure, diastolic blood pressure, and mean arterial pressure. Although there were no differences in serum insulin concentrations, the LFC group had lower fasting blood glucose than either the HFP or HFPn3 groups. The high fasting blood glucose concentrations of pigs fed the HFP or HFPn3 diets caused an increase in HOMA-IR as compared with the LFC group.

Also summarized in **Table 4**, there were no differences in serum CRP or TNFα attributable to dietary treatment. However, serum CRP was positively correlated with fasting blood glucose ($r=0.42716$, $p=0.0421$). IL-6, IL-10, and IL-12 were detectable in the serum of only two pigs (data not shown). Serum adiponectin was decreased by both high fat diets vs. the control group, but the magnitude of this decrease was attenuated in pigs fed the HFPn3 diet (**Figure 1A**). Serum adiponectin was negatively correlated with fasting blood glucose ($r=-0.44268$, $p=0.0287$).

To assess whether differences in serum adiponectin were associated with altered AMPK activity, we measured LD muscle AMPKα phosphorylation. Both the HFP and HFPn3 groups had decreased phosphorylation of AMPKα at Thr_{172}, and a decreased ratio of Thr_{172} AMPKα to total AMPKα as compared with the LFC group, but did not differ in total

AMPKα (**Figure 1B-D**). Phosphorylation of AMPKα at Thr_{172} was positively correlated with serum adiponectin concentration (r=0.47112, p=0.0233) and negatively correlated with fasting blood glucose (r=-0.49803, p=0.0156). Additionally, we determined whether there was a dietary influence on ectopic lipid deposition. Percent lipid in liver and LD muscle was not influenced by diet (**Table 3**).

Insulin sensitivity was assessed by measuring the decrease in blood glucose following an insulin challenge. Blood glucose was decreased by 5 min in the LFC group, and by 10 min in the group fed the HFPn3 diet (**Figure 2**). The HFP group was unresponsive to insulin over the 15 min insulin challenge (**Figure 2**). Tissue differences in insulin signaling were assessed by evaluating phosphorylation of PKB at Ser_{473}. The HFPn3 diet decreased PKB abundance in SQ, VIS, and OM adipose depots (**Figure 3A, 3D, 3G**). However, there was no difference is Ser_{473} phosphorylation of PKB (**Figure 3B, 3E, 3H**). The ratio of Ser_{473} PKB to total PKB was decreased in the SQ and OM depots by the HFP diet, but not the HFPn3 diet (**Figures 3C, 3I**), but was unchanged in the VIS depot (**Figure 3F**). There was no difference in liver or LD muscle total PKB, or PKB phosphorylation (data not shown). Adipose tissue macrophages were evaluated by flow cytometry to determine both the extent of macrophage infiltration (CD203a) and phenotype. Phenotype was determined by the presence of CD16, an Fcγ receptor, and CD14, the co-receptor for tlr4. The percent of macrophages in adipose depot SVC was decreased in the HFP and HFPn3 groups with no difference amongst adipose depots (**Figure 4A**).

The HFP and HFPn3 groups had increased $CD16^+$ macrophages in the adipose tissue compared with the LFC group, with no difference among the adipose depots (**Figure 4B**). There was no difference in $CD16^+$ $CD14^+$ macrophages among dietary treatments; however,

the OM adipose depot had decreased $CD16^+$ $CD14^+$ macrophages compared to the SQ adipose depot (**Figure 4C**). Additionally, the HFP diet increased the percent $CD16^-$ $CD14^+$ macrophages compared with the LFC and HFPn3 groups (**Figure 4D**) with no differences across adipose depots.

Discussion

Comparative animal models are critical to understanding the development of human obesity and the chronic inflammation in adipose tissue which contributes to the pre-diabetic state (i.e., metabolic syndrome). Although previous studies (11; 24; 33-34) have underscored the potential value of the Ossabaw pig as a model for obesity and pre-diabetes, this is to our knowledge the first study to address obesity-linked inflammation in adipose tissue of the Ossabaw pig, or the potential alleviation of this inflammation by dietary n-3 fatty acids. Whereas others (20) have reported increased circulating concentrations of TNFα in response to diet-induced obesity in Ossabaw swine, we found no diet-related increase in serum concentrations of common pro-inflammatory markers, TNFα, CRP, IL-6, or IL-12, despite the prolonged duration of our study and the marked development of obesity (i.e., over 100 kg weight gained beyond mature body weight). Whether this difference relates to differences in diet composition across studies is currently unresolved. It is noteworthy that whereas others (20) have attributed both inflammation and liver injury to the presence of fructose in atherogenic diets, our diet contained sucrose which contributed 14% fructose to the diet on a weight basis), and no added cholesterol. However, it is also intriguing to note that reduced ectopic storage of lipid in liver and skeletal muscle due to increased capacity for expansion of adipose mass has been associated with improvements in metabolic markers associated with obesity (17), and presumably, alleviation of obesity-linked inflammation. We saw no

evidence of ectopic lipid accumulation in the liver or skeletal muscle of pigs fed either high fat diet. Consequently, the marked capacity of the Ossabaw swine used in our study to expand adipose mass may in fact afford them some protection against obesity-linked inflammation.

To evaluate adipose inflammation in this model and make comparisons with human literature, we investigated the adipose tissue macrophage population in lean vs. obese Ossabaw swine. Several studies of human adipose tissue have categorized macrophages as being $CD14^+$ (4; 18; 35; 41). In our study, only a small percentage (8-10%) of the adipose-derived SVC were identified as $CD14^+$. Consequently, we used the mature macrophage marker, CD203a, to identify macrophage populations and the markers CD14 and CD16 to assess phenotypic changes associated with diet or adipose depot. Surprisingly, both the HFP and HFPn3 groups had fewer macrophages than the LFC group, although this decrease did not occur in all depots across diets. It seems possible that accelerated macrophage turnover in obese pigs led to an increase in adipose monocytes not expressing CD203a. It is also possible that recruitment of adipogenic progenitors, due to rapid expansion of adipose mass in pigs fed the high fat diets, decreased the overall percentage of macrophages in the SVC population.

The predominant population of adipose macrophages in the Ossabaw pig were $CD16^+$, with the vast majority of these being $CD14^-$. This is quite intriguing as there is a paucity of information relating to this particular phenotype of adipose macrophages. Given that $CD16^+CD14^-$ monocytes are anti-inflammatory (7), it is quite possible that adipose inflammation in obese Ossabaw swine may be minimized by the majority of macrophages being of the $CD16^+CD14^-$ phenotype. However, it is important to note that there was in fact

an increase in the number of $CD16^{-}CD14^{+}$ macrophages detected in the adipose tissue of pigs fed the HFP diet vs. the LFC or HFPn3 groups. In humans, classical $CD16^{-}CD14^{+}$ blood monocytes express high levels of CCR2 and CD62L (29), and are thus pro-inflammatory (7). This perhaps indicates that the greater number of $CD16^{-}CD14^{+}$ macrophages in the adipose tissue of pigs fed the HFP diet reflects obesity-linked infiltration with pro-inflammatory cells, and that the HFPn3 diet protected against this infiltration, despite the marked obesity. It is noteworthy, that we have previously demonstrated that dietary supplementation with n-3 fatty acids causes enrichment of both adipose tissue and muscle (14), and thus higher concentrations of n-3 PUFA in the adipose tissue of pigs fed the HFPn3 diet may have protected them from the increase in the $CD16^{-}CD14^{+}$ macrophages.

As regards indicators of pre-diabetes, fasting hyperglycemia was readily induced by both high fat diets, and this occurred with increased HOMA-IR and blood pressure measures. Hyperglycemia and hyperinsulinemia have been reported (19-20) in pig models, although insulin peaked at one month and decreased thereafter in one study (19). The degree of hyperglycemia induced by the high fat diets without concomitant hyperinsulinemia is striking. There are instances where hypoinsulinemia is present in a diabetic state (9), and may be caused decreased beta cell function due to chronic hyperglycemia (10). However, this has not been addressed in this model to our knowledge. Although, cholesterol and triglycerides have also been increased by high fat diets in several studies utilizing swine (11; 19-20; 24), the diets used in our study did not cause significant increases in either. It should be noted that we did not add cholesterol to the diets as other researchers have done (11; 20; 24). In Ossabaw swine, dietary cholesterol likely plays a pivotal role in the development of hypercholesterolemia and hypertriglyceridemia. Circulating NEFA were increased in only in

the HFPn3 group vs. the LFC group. This is most likely a consequence of the slightly higher fat content of the HFPn3 diet due to addition of the n3 fatty acid source.

In the context of obesity, the activity of AMP-activated protein kinase (AMPK) is quite important due to its stimulation of glucose uptake and fatty acid oxidation in muscle (31; 40). Relative to the control group, we found that both obese groups had decreased abundance of phosphorylated Thr_{172} AMPKα and a decrease in the ratio of phosphoThr_{172} AMPKα to total AMPKα in the LD muscle. Genetically, Ossabaw swine contain the mutation $Val_{199}\rightarrow$Ile in the PRKAG3 (the γ_3 isoform of AMPK) gene which negatively affects the activity of AMPK (22). It has been hypothesized that decreased AMPK activity due to this mutation is causal in the development of insulin resistance in Ossabaw pigs (27). Although adiponectin clearly stimulates activation of AMPK (40), it seems unlikely that the relatively small decrease in serum adiponectin in the obese groups vs. the control group contributed to the reduction in Thr_{172} AMPKα. Given the PRKAG3 mutation, it will indeed be intriguing to determine whether Ossabaw pigs are more susceptible to the effects of high fat diets on AMPK activity than are pigs of commercial lean lines.

Changes in blood glucose concentrations during the insulin challenge indicated that n-3 fatty acid supplementation improved insulin sensitivity vs. pigs fed the HFP diet. However, there was no dietary difference in Ser_{473} PKB in adipose tissue, liver, or LD muscle after insulin stimulation. Total PKB was decreased in all adipose depots compared to the LFC group and Ser_{473} PKB:PKB was increased in SQ and OM adipose depots in pigs fed the HFPn3 diet compared to the HFP pigs. In adipocytes, activation of peroxisome proliferator-activated receptor (PPAR)γ by thiazolidinediones decreases PKBα abundance specifically (38). Previous work has shown that n3 fatty acids (or their metabolites) are

ligand activators of PPARγ (3; 25) and it is quite possible that the reduction in PKB reflects this activity of n-3 fatty acids in adipocytes. Nonetheless, the reduction in PKB did not preclude the stimulation of glucose uptake by insulin during the insulin challenge. Additionally, it should be noted that the insulin challenge was performed under anesthesia, which although necessary for this study, is also limitation.

In summary, we have shown marked obesity, hyperglycemia, hypertension and a modest reduction in circulating adiponectin concentrations in Ossabaw swine fed a diet high in saturated fat. However, there were only limited indications of the expected obesity-linked inflammation in adipose tissue. The predominate evidence of inflammation was the increased $CD16^{-}CD14^{+}$ macrophages in the adipose tissue of the HFP group, which are most likely pro-inflammatory macrophages. Despite the lack of a clear inflammatory response, the HFP group had decreased sensitivity to administration of exogenous insulin as compared with the control and HFPn3 groups. Major effects of n-3 fatty acid supplementation were determined to be the regulation of PKB abundance, improved insulin-stimulated glucose clearance during the insulin challenge, and blunted infiltration of adipose tissue with $CD16^{-}CD14^{+}$ macrophages. Further investigation with the monocytic markers CD172a and CD163 may provide further insight to adipose depot macrophage populations in this novel model of obesity and pre-diabetes. Finally, the loss of three pigs from the HFPn3 dietary treatment, although not definitively linked to diet, limited our statistical power in studying the beneficial effects of n-3 PUFA in this model.

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Tables

Table 2. *Ingredient composition of the diet (%) as-fed.*

Ingredient	Diet		
	LFC	**HFP**	**HFPn3**
Corn	46.92	25.06	13.32
Soybean Meal-48	4.00	19.55	21.35
Sucrose	-	28.00	28.00
Palm Oil	-	18.00	18.00
Menhaden Meal	7.50	-	-
Soybean Hulls	33.44	-	-
Wheat Bran	3.00	-	-
Canola Oil	1.00	-	3.00
Gromega™ 365[1]	1.00	-	5.00
Meat & Bone Meal[2]	-	6.25	6.25
Vitamin Mix	0.50	0.50	0.50
Salt	0.40	0.40	0.40
Mineral Mix	0.10	0.10	0.10
Selenium Mix	0.05	0.05	0.05
DL-Methionine	-	0.07	0.09
Dicalcium Phosphate	1.88	1.63	1.64
Limestone	0.21	0.38	2.30
Ethoxyquin[3]	0.0125	0.0125	0.0125
Metabolizable Energy (kcal/kg)	2750	4059	4058
Fat (kcal/kg)	382	1572	1877
Ether Extract (%)	4.69	20.27	23.98
Crude Protein (%)	14.92	15.14	15.00
Lysine (%)	0.81	0.80	0.83
Methionine (%)	0.29	0.29	0.29
Met + Cys (%)	0.47	0.50	0.50
Crude Fiber (%)	13.69	1.39	1.19
Calcium (%)	0.90	1.20	1.93
Available Phosphorous	0.60	0.60	0.60

[1]JBS United, Sheridan, IN; [2]Darling International, Des Moines, IA; [3]Novus International, St.

Table 3. *Dietary fatty acid profile (g/100 g total fatty acid).*

Fatty Acid	Diet		
	LFC	**HFP**	**HFPn3**
6:0	0.00	0.00	0.00
8:0	0.00	0.00	0.00
10:0	0.66	0.80	0.75
12:0	0.00	1.44	2.12
14:0	2.34	1.06	1.59
14:1	0.00	0.00	0.00
16:0	18.47	43.98	38.85
16:1	2.51	0.00	0.89
18:0	4.08	5.18	4.83
18:1	28.14	36.6	37.58
18:2n6	34.14	10.73	10.49
18:3n6	0.00	0.00	0.00
18:3n3	3.77	0.23	1.03
20:0	0.44	0.00	0.21
20:1	0.66	0.00	0.00
20:2	0.00	0.00	0.00
20:3	0.00	0.00	0.00
20:3n6	0.00	0.00	0.00
20:4n6	0.00	0.00	0.00
20:5n3	2.33	0.00	0.88
22:1	0.00	0.00	0.00
22:2	0.00	0.00	0.00
24:0	0.00	0.00	0.00
22:6n3	2.10	0.00	0.78
Other	0.37	0.00	0.00
Total	100.00	100.00	100.00
Saturated	26.36	52.45	48.35
n3	8.20	0.23	2.69
n6	34.14	10.73	10.49
n6/n3	4.16	47.59	3.91

Table 4. *Growth and metabolic parameters of Ossabaw swine at termination.*

Parameter	Diet			p-value
	LFC	HFP	HFPn3	
Final Body Weight (kg)	49.6±4.7[a]	156.7±5.3[b]	154.4±5.8[b]	<0.0001
Insulin (pg/mL)	63.5±8.6	80.3±11.7	76.5±14.7	0.44
Blood Glucose (mg/dL)	119.8±14.8[a]	233.5±29.4[b]	221.5±34.4[b]	0.0011
HOMA-IR	0.44±0.12[a]	1.03±0.13[b]	0.97±0.18[b]	0.0121
Cholesterol (mg/dL)	68.4±5.7	90.8±6.4	79.0±6.9	0.06
Triglycerides (mg/dL)	42.0±7.1	61.9±7.4	61.4±8.7	0.11
Liver Lipid (%)	4.23±0.18	4.54±0.18	4.58±0.22	0.39
LD Lipid (%)	3.53±1.06	5.90±1.10	5.98±1.29	0.21
NEFA (mM)	0.57±0.22[a]	1.13±0.23[ab]	1.46±0.27[b]	0.0453
CRP (ng/mL)	13.7±2.5	20.6±2.6	18.4±3.0	0.17
TNFα (pg/mL)	24.9±3.2	21.6±2.8	15.5±2.3	0.10
Homocysteine (μM)	11.5±5.8	33.0±5.2	26.2±8.1	0.06
Diastolic BP	63.4±7.9[a]	96.3±7.1[b]	102.0±9.0[b]	0.0251
Systolic BP	127.2±6.9[a]	170.1±6.3[b]	178.7±8.1[b]	0.0021
Mean Arterial Pressure	93.6±8.9[a]	118.0±7.9[b]	134.0±10.2[b]	0.0275

Figures

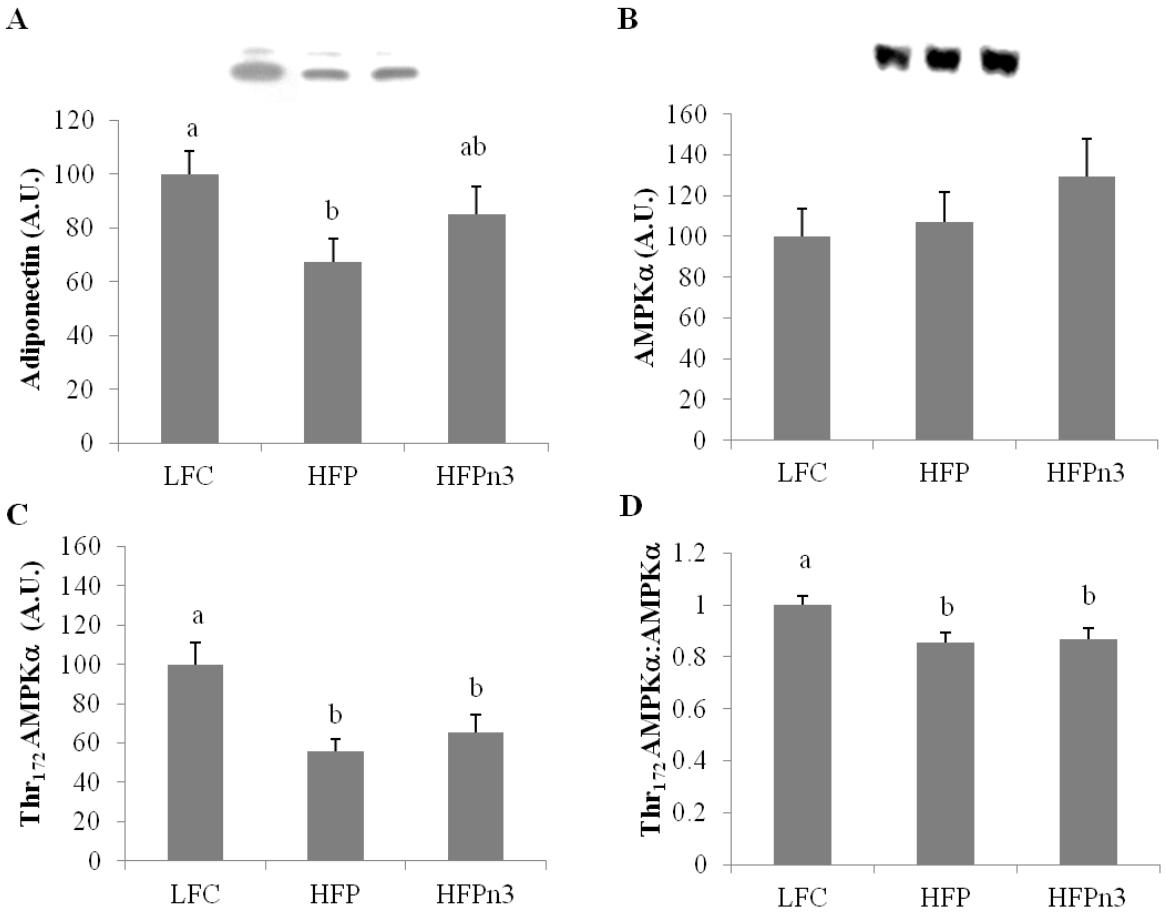

Figure 1. *n-3 fatty acids attenuate the high palm oil induced decrease in serum adiponectin, but do not alter AMPK.*

(A) Relative serum adiponectin and (B) AMPKα, (C) Thr_{172} AMPKα, and (D) Thr_{172} AMPKα:AMPKα of LD Ossabaw swine fed low fat control (LFC), high fat palm oil (HFP), or high fat palm oil plus n-3 fatty acids (HFPn3) diets. Values are (A-B) arbitrary densitometric units or (C) arbitrary units per mg protein.. Means with different letters represent a difference of $p<0.05$. Main effects (A) Diet p=0.0410; (B) Diet p=0.42; (C) Diet=0.0055; (D) Diet p=0.0136.

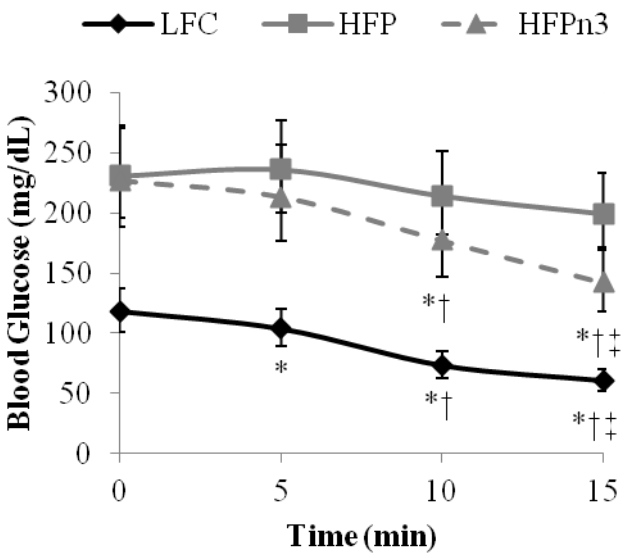

Figure 2. *n-3 fatty acids attenuate high palm oil induced insulin resistance.*

Blood glucose from Ossabaw swine challenged with 0.25 IU/kg porcine insulin after a 12 hour fast from 3 dietary treatments: low fat control (LFC), high fat palm oil (HFP), high fat palm oil plus n-3 fatty acids (HFPn3). Differences are from 0 to 5 min, 5 to 10 min, and 10 to 15 min within diet are represented by *, †, and ‡, respectively. Main effects: Diet p=0.0004, Time p=<0.0001, Diet*Time p=0.0481.

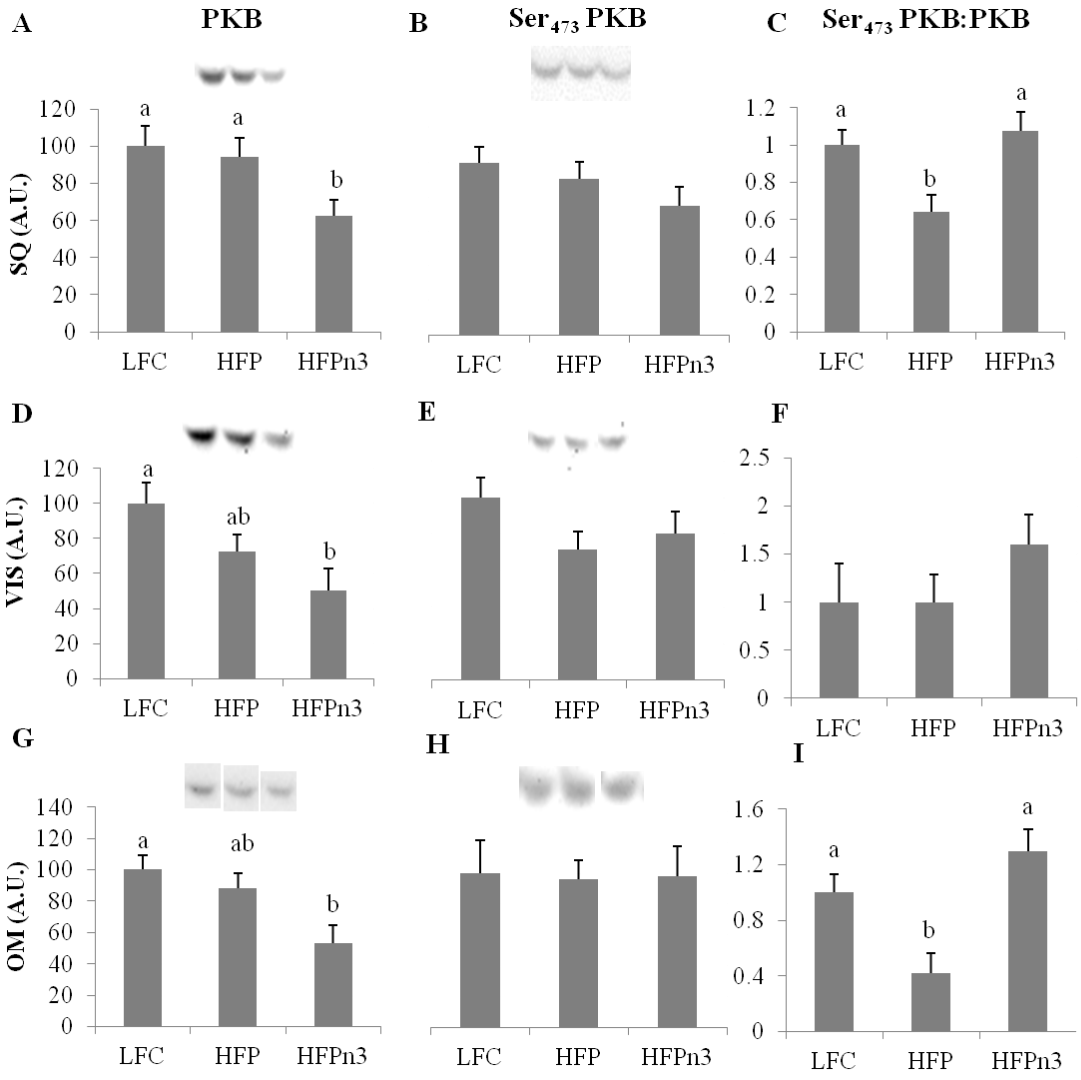

Figure 3. *Insulin resistance is not reflected in adipose tissue S_{473} PKB phosphorylation.*

(A) PKB, (B) Ser_{473} PKB, and (C) Ser_{473} PKB:PKB of subcutaneous adipose (SQ), (D) PKB, (E) Ser_{473} PKB, and (F) Ser_{473} PKB:PKB of visceral adipose (VIS), and (G) PKB, (H) Ser_{473} PKB, and (I) Ser_{473} PKB:PKB of omental adipose (OM) from Ossabaw swine fed low fat control (LFC), high fat palm oil (HFP), or high fat palm oil plus n-3 fatty acids (HFPn3) diets. Values are arbitrary densitometric units. Means with different letters represent difference of $p<0.05$. Main effects: (A) Diet p=0.0238; (B) Diet=0.34; (C) Diet p=0.0141; (D) Diet p=0.0326; (E) Diet=0.18; (F) Diet p=0.22; (G) Diet p=0.0153; (H) Diet=0.12; (I) Diet p=0.0032.

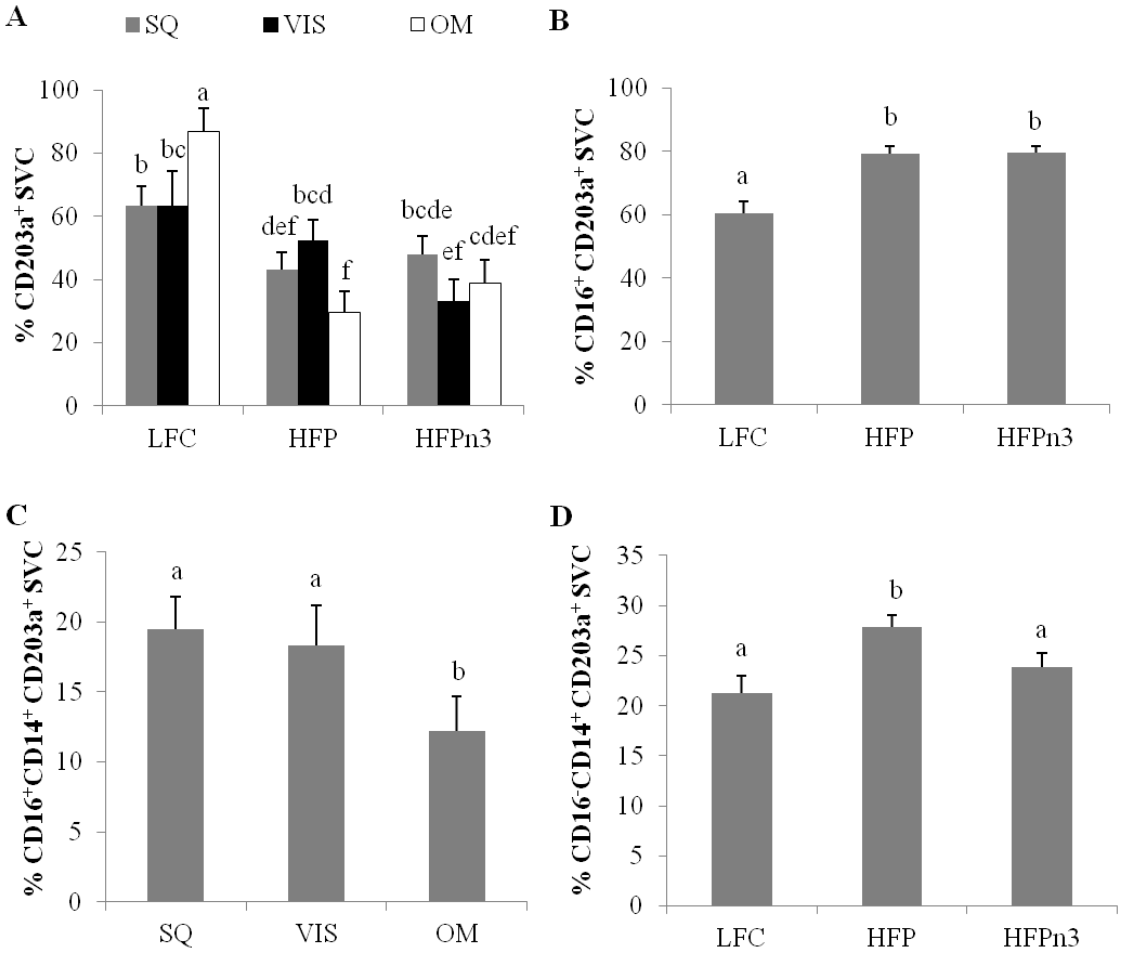

Figure 4. *High fat diets have fewer macrophages, but phenotype is altered by obesity and depot.*

(A) Macrophages (CD203a), and (B) $CD16^+$ macrophages by diet, (C) CD16+$CD14^+$ macrophages by adipose depot, and (D) $CD16^-$ $CD14^+$ macrophages by diet in subcutaneous (SQ), visceral (VIS), and omental (OM) adipose depots from Ossabaw swine fed low fat control (LFC), high fat palm oil (HFP), or high fat palm oil plus n-3 fatty acids (HFPn3) diets. Values are percent positive out of SVC's. Means with different letters represent difference of $p<0.05$. Main effects: (A) Diet p=0.0002, Tissue p=0.9192, Diet*Tissue p=0.0007; (B) Diet p=p<0.0001, Tissue p=0.10, Diet*Tissue p=0.21; (C) Diet p=0.50, Tissue p<0.0443, Diet*Tissue p=0.98; (D) Diet p=0.0053, Tissue p=0.65, Diet*Tissue p=0.74.

CHAPTER 4: ADIPOGENIC POTENTIAL OF OSSABAW ADIPOCYTES PROTECTS AGAINST IL-8 SECRETION

Abstract

Some obese individuals appear to be protected against obesity-linked adipose tissue inflammation and the ensuing metabolic consequences. This protection may be afforded by an inherent ability to markedly expand adipose tissue by hyperplasia in response to excess caloric intake, as it has been previously demonstrated that adipose tissue expansion improves both the metabolic and inflammatory profile. It was determined that Ossabaw swine develop minimal evidence of inflammation, despite morbid obesity induced by long-term consumption of a high fat diet. The ability of Ossabaw swine to store large amounts of lipid in adipose tissue when provided excess calories may have provided protection against the development of inflammation. Thus, we hypothesized that Ossabaw adipocytes will have increased adipogenesis and be protected against palmitate-induced inflammation and insulin resistance compared with Yorkshire adipocytes. Ossabaw adipocytes have increased clonal expansion, PPARγ expression, and G3PDH activity compared to Yorkshire adipocytes. Palmitate activated NFκB, but activation was not different between Ossabaw and Yorkshire adipocytes. However, palmitate only induced IL-8 production in Yorkshire adipocytes. The increased adipogenic potential of Ossabaw adipocytes may be protective against palmitate induced IL-8 production. This is confirmed by the lack of obesity-linked increases in serum and adipose tissue IL-8 concentrations in Ossabaw swine. As determined by 2-NBDG uptake, insulin sensitivity was decreased by both palmitate, irrespective of genotype. However, Ossabaw adipocytes had both lower basal and insulin-stimulated 2-NBDG uptake.

Thus, the *in vitro* evidence supports that the adipogenic potential of Ossabaw adipocytes may be protective against palmitate induced inflammation with respect to IL-8 production.

Introduction

The prevalence of obesity is increasing in the United States (16). Obesity is considered a state of chronic low-grade inflammation. Nearly all of the co-morbidities associated with obesity can be linked to inflammation (3; 29). It is estimated that ~80% of individuals with type 2 diabetes (31) and 65% of individuals with the metabolic syndrome (15) are obese. Some obese individuals appear to be protected against obesity-linked inflammation and metabolic disturbances (22; 38). These individuals are termed metabolically healthy but obese (MHO). MHO individuals have decreased inflammation compared to their un-healthy counterparts, including lower C-reactive protein (CRP) (21; 37), interleukin 6 (IL-6) (24; 37), and tumor necrosis factor (TNF) α (24) as well as increased adiponectin (1; 44).

In addition to decreased inflammation, MHO individual have a more favorable metabolic profile. Compared with their un-healthy obese counterparts, MHO individuals have less visceral fat (7; 18; 20-21; 24; 44) and less ectopic lipid deposition in muscle (39; 44) and liver (39), despite similar BMI (7; 18; 20-21; 37) and body fat percentage (7; 21). Additionally, MHO individuals have decreased circulating concentrations of triglycerides (7; 18; 20-21; 24; 37), total cholesterol (24), LDL-cholesterol (24; 37), and increased concentrations of HDL-cholesterol (7; 21; 24; 37) compared to their un-healthy counterparts. MHO individuals retain insulin sensitivity (7; 18; 20-21; 37; 39) and have lower fasting insulin (7; 20-21) and glucose (7; 18; 20).

An extraordinary ability to expand subcutaneous adipose tissue may be a fundamental component of the favorable metabolic profile exhibited by MHO individuals. Evidence suggests that adipose tissue has a genetic limit on its ability to expand by hypertrophy and hyperplasia; and that the ability to expand varies by depot (12). Excess energy intake may exhaust the body's ability to store fat in adipose tissue, leading to ectopic lipid deposition in the muscle and liver. Ectopic lipid deposition has been closely linked to the development of insulin resistance (45). In *ob/ob* mice (devoid of leptin), the induction of adipose tissue expansion by over-expression of adiponectin attenuates insulin resistance and improves systemic inflammation normally seen in this model, despite morbid obesity (23).

Swine are increasingly being used as a biomedical model for researching obesity, insulin resistance, and cardiovascular disease (8; 10; 26-27; 30; 35). Ossabaw swine are an interesting model for studying obesity and its co-morbidities due to the breed's ability to accumulate lipid, develop impaired glucose tolerance, and frequent development of insulin resistance (28; 30; 42-43). However, we recently found that when fed a high fat diet for 30 weeks, Ossabaw swine display little evidence of the expected chronic low-grade inflammation associated with morbid obesity (14). Potentially, the ability of Ossabaw swine to accumulate adipose tissue may protect them against the development of inflammation. Thus, we hypothesized that the ability of Ossabaw swine to expand adipose tissue more readily than lean commercial swine will be evident by increased adipogenesis in Ossabaw stromal vascular cell (SVC) derived adipocytes and that they will have decreased palmitate-induced inflammation and insulin resistance compared to Yorkshire SVC derived adipocytes.

Methods

Animals. All animal procedures were conducted at Iowa State University and were approved by the Iowa State University Institutional Animal Care and Use Committee. To demonstrate the ability of Ossabaw swine to expand adipose tissue, subcutaneous (SQ) and visceral (VIS) adipose tissue was collected from a study previously described (14). Briefly, 27 mature male and female Ossabaw swine were fed three diets for 30 weeks: a restricted Low Fat Control (LFC), an *ad libitum* High Fat Palm Oil (HFP), and an *ad libitum* High Fat Palm Oil plus n-3 fatty acids (HFPn3). Final body weights for the three diets were 49.6, 156.7 and 154.4 kg, respectively. Additionally, SQ adipose tissue was collected from Yorkshire swine (~110 kg) to compare adipocyte size.

Adipocyte Size. Frozen sections of adipose depots were placed in 10% neutral buffered formalin overnight and then embedded in paraffin. Thereafter, sections of 5 μm thickness were mounted onto microscope slides for staining with hematoxylin and eosin for analysis of adipocyte area and adipocyte number. Slides were imaged with a LEICA DM1 3000B microscope (LEICA Microsystems Inc., Buffalo Grove, IL) equipped with a QICAM Fast 1394 camera (QIMAGING, Surrey, BC, Canada). Adipocyte area was analyzed from an average of 6 fields at 10X magnification with AxioVision LE 4.8.2 (Carl Zeiss AG, Thornwood, NY). Measurements were taken from duplicate slides.

Cell Culture. SVC were isolated from lean Ossabaw and Yorkshire swine as previously described (14). Cells were grown in LG-DMEM (pH 7.4) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL Streptomycin, 5 μg/mL gentamicin, and 2.5 μg/mL Amphotericin B. Media was changed every two days during the growth phase until one day after confluency. For differentiation of SVC into adipocytes, growth media was

supplemented with 100 nM insulin, 0.1% ITS (Sigma-Aldrich, St. Louis, MO), 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 33 μM biotin, 17 μM pantothenic acid, and 5 μM troglitazone for 6 days. Thereafter, the media was changed at days 6 and 8 to growth media supplemented with 100 nM insulin 0.25 μM dexamethasone, 33 μM biotin, and 17 μM pantothenic acid. All experiments utilized SVC between passages 3 and 6.

SVC Proliferation and Differentiation. SVC growth phase proliferation was determined for 3 days using BrDU incorporation (Millipore, Billerica, MA). Additionally, clonal expansion was measured for the first 3 days of differentiation using BrDU incorporation (Millipore). At days 10 days post-differentiation, adipocytes were stained with Oil Red O as follows: Cells were fixed for one hour with 10% neutral buffered formalin. Then were washed 3X with 60% isopropanol and stained for 10 minutes in a 0.21% w/v Oil Red-O in 60% isopropanol. Cells were then washed 4X with tap water and covered with 50% glycerol. Images were taken as described above for quantification of lipid droplet size. Cells were then washed 4X with tap water and air dried. Oil Red O was extracted with 100% isopropanol for 30 minutes with rocking and was quantified by measuring the absorbance at 500 nm.

G3PDH. The enzyme activity of glycerol-3-phosphate dehydrogenase (G3PDH) was determined from 10 day post-differentiation Ossabaw and Yorkshire SVC cell lysate. The assay for G3PDH was performed as described by Gamou et al. (17). Briefly, cell lysate was added to 100 μL of reaction buffer (125 mM Triethanolamine-HCl pH 7.5, 2.5 mM EDTA, 0.5 mM NADH, 1.1 mM dihydroxyacetone phosphate, 0.125 mM β-mercaptoethanol) and the absorbance was read at 340 nm. Reagents for G3PDH activity were obtained from

Sigma-Aldrich (St. Louis, MO). Absorbance measurements were taken at 340 nm every 30 s for 7 min at 25°C on Synergy 4 microplate reader (Bio-Tek, Winooski, VT). G3PDH activity was calculated from the change in absorbance at 340 nm and expressed as nmol NAD(P)H per min per mg protein.

Insulin Sensitivity. Ossabaw and Yorkshire adipocytes (10 day post differentiation) were treated with Vehicle (untreated), 0.5 mM sodium palmitate, or 10 μg/mL LPS (*Escherichia coli* serotype O55:B55) in LG-DMEM (pH 7.4) with 0.1 mM BSA, 100 U/mL penicillin, 100 μg/mL Streptomycin for 48 hours with a media change at 24 hours. At 48 hours, cells were washed once with PBS, and then incubated with or without 100 nM insulin for 15 minutes. Thereafter 2-NBDG (2-deoxy-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose) (Cayman Chemical, Ann Arbor, MI) was added at a final concentration of 100 μg/mL and incubated for 30 minutes. Cells were washed twice with PBS and the uptake of 2-NBDG was measured by fluorescence with the excitation/emission at 485/535 nm. Cellular protein was collected and protein was measured using the BCA reagent (Pierce, Rockford, IL). Total cellular protein was used as a covariate for 2-NBDG uptake.

Western Blot Analysis. Western blots were performed as previously described (14) with protein isolated from palmitate and LPS treated adipocytes. Nitrocellulose membranes were probed with PPARγ rabbit polyclonal antibody (Millipore).

ELISA. Nuclear Factor κ B p 65 nuclear translocation was measured on palmitate and LPS treated cells using an ELISA (Cayman Chemical). Nuclear Extracts were extracted according to Cayman Chemical protocol with modifications. Protease and phosphatase inhibitors used in the extractions were 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride,

5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin for both the hypotonic buffer and the nuclear extraction buffer. Media IL-8 and IL-6 were measured on 48 hour cell culture treatment media (R&D Systems, Minneapolis, MN). Additionally, IL-8 concentration was determined on serum and SQ and VIS adipose tissue lysate from lean and obese Ossabaw swine.

Myeloperoxidase Activity. Myeloperoxidase activity was determined in serum using previously described methods (25; 40) with modifications. Adipose tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0) with 0.5% (w/v) hexadecyltrimethylammonium bromide and freeze thawed and vortexed three times. Samples were then centrifuged for 10 minutes at 10,000 x g and the supernatants were collected. Adipose tissue supernantants and serum were mixed with o-dianasidine dihydrochloride and 0.005% (v/v) hydrogen peroxide. The oxidation of o-dianasidine was measured by the change of absorbance over a 10 minute period at 460 nm and 25°C. One unit of myeloperoxidase activity was defined as that degrading one μmol of hydrogen peroxide per min at 25°C. A molar extinction coefficient of 1.3×10^4 M^{-1} cm^{-1} was used for determining myeloperoxidase activity.

Statistical Analysis. All statistical procedures outlined were performed using SAS 9.2 (SAS, Cary, NC). Residuals were analyzed to detect outliers and normality using the PROC Univariate procedure. Right-skewed data were log-transformed for further analysis. ANOVA was performed using the PROC MIXED procedure. Main effects for Ossabaw tissue data included lean (LFC) and obese (HFP and HFPn3) and tissue where appropriate. Fixed effects include sex, group, and termination date. Fixed effects were used in the model when $p \leq 0.15$ for the fixed effect or interaction with main effect(s). Cell culture data for

Western and NFκB ELISA analysis were done with a cell culture lot containing an even mixture of SVC from 3 pigs from the respective breed. All other data was done on three separate SVC from each breed unless otherwise noted. SVC replicate was included in the model when $p \leq 0.15$ for the fixed effect or interaction with swine breed. Data presented are least-squares means ± standard error. Data was considered statistically different when $p < 0.05$.

Results

To assess the ability of Ossabaw swine to accrue adipose tissue, we measured adipocyte size in SQ and VIS adipose tissue depots (**Figure 5**). Swine fed high fat diets (i.e. HFP and HFPn3) had marked enlargement adipocytes compared to the LFC fed swine SQ and VIS depots, with no difference between adipose depots. To verify comparable adipocyte size of lean Ossabaw and Yorkshire swine, we also determined adipocyte size in Yorkshire SQ adipose tissue. There was no difference in LFC Ossabaw and Yorkshire SQ adipose tissue adipocyte size (data not shown). Thus, our results demonstrate that Ossabaw swine have a marked capacity to store excess energy through adipocyte hypertrophy. Additionally, in a lean state, Ossabaw and Yorkshire swine do not differ in adipocyte size.

To compare Ossabaw and Yorkshire adipocytes *in vitro*, SVC from lean Ossabaw and Yorkshire SQ adipose tissue were proliferated and differentiated into adipocytes. Yorkshire SVC cells proliferated faster during cell expansion (**Figure 6A**). Thus, to ensure comparable cell populations, differentiation was not initiated until one day post cell confluence. During the initial stages of differentiation into adipocytes, Ossabaw SVC had increased clonal expansion compared to Yorkshire SVC (**Figure 6B**); representing an increased capacity for adipose expansion due to preadipocyte hyperplasia. Concomitant with the increased clonal

expansion, Ossabaw adipocytes also had increased enzymatic activity of G3PDH (**Figure 6C**), a marker of adipogenesis. Despite increased clonal expansion and G3PDH activity, Ossabaw adipocytes had decreased uptake of Oil Red O (**Figure 7**), and therefore decreased lipid filling compared to Yorkshire adipocytes. In addition to clonal expansion and G3PDH activity, PPARγ1, but not PPARγ2 or total PPARγ, was increased in Ossabaw compared to Yorkshire adipocytes (**Figure 8**).

A glucose analogue, 2-NBDG, was used to test the effect of palmitate and LPS treatments on insulin sensitivity in Ossabaw and Yorkshire adipocytes (**Figure 9**). Insulin increased 2-NBDG uptake in both Ossabaw and Yorkshire adipocytes. Yorkshire adipocytes had both increased basal and insulin-stimulated 2-NBDG uptake compared to Ossabaw adipocytes. Both palmitate and LPS treatment induced insulin resistance compared vehicle treatment. However, palmitate and LPS did not differentially alter insulin resistance in either adipocyte genotype. In spite of the increased basal and insulin-stimulated 2-NBDG uptake displayed by Yorkshire adipocytes, both genotypes had a ~1.5 fold increase in 2-NBDG uptake following insulin stimulation.

Nuclear translocation of NFκB p65 was determined in Ossabaw and Yorkshire adipocytes (**Figure 10A**). NFκB nuclear translocation was increased by palmitate and LPS in both adipocyte genotypes. However, there was no difference in NFκB translocation between Ossabaw and Yorkshire adipocytes. Cellular PPARγ content was determined by western blot (**Figure 8**). Total PPARγ, PPARγ1, and PPARγ2 content was not altered by treatment (i.e. V, P, L), however as mentioned above, Ossabaw adipocytes had increased PPARγ1 compared Yorkshire adipocytes.

In the media, IL-8 production by adipocytes was differentially regulated by treatment (i.e. V, P, L), with Yorkshire adipocytes producing significantly more IL-8 than Ossabaw adipocytes (**Figure 10B**). Both Ossabaw and Yorkshire adipocytes had increased IL-8 production in response to LPS, but only Yorkshire adipocytes responded to palmitate with increased IL-8 production. Yorkshire and Ossabaw adipocytes did not differ in IL-6 excretion, nor did either genotype produce IL-6 in response to palmitate (**Figure 10C**). However, like IL-8 Ossabaw adipocytes produced more IL-6 in response to LPS than Yorkshire adipocytes.

Since palmitate induced IL-8 production in Yorkshire, but not Ossabaw adipocytes, we determined whether there was any high fat diet-induced increase in serum or adipose tissue IL-8 in Ossabaw swine. There was no diet-induced increase in serum IL-8 concentrations (**Figure 11A**). Additionally, there were no diet or adipose tissue differences in IL-8 concentrations (**Figure 11B**). Although there was a significant diet*tissue interaction, with obese VIS adipose tissue having the highest IL-8 concentrations, and lean VIS adipose tissue have the lowest concentrations. Since IL-8 is a chemokine that causes neutrophil extravasation, we determined myeloperoxidase activity in serum and SQ and VIS adipose tissue. There were no differences between lean and obese pigs in serum myeloperoxidase activity (**Figure 11C**). Furthermore, myeloperoxidase activity was not detected in any adipose tissue depot of any pig.

Discussion

As evidenced by Faris *et al.* (14) and others (4; 6; 27), Ossabaw swine gain considerable weight when fed excess calories. We have demonstrated that this excessive body weight gain leads to a ~4.3 and ~8.0 fold increases in SQ and VIS adipocyte size,

respectively. Adipocyte hypertrophy in obese Ossabaw swine is much greater in magnitude compared to the ~2 to 3 fold increase in obese human (36) and rodents (25). Despite apparent obesity and adipocyte hypertrophy in SQ and VIS adipose tissue, Ossabaw swine did not display any evidence of systemic inflammation, and only a modest increase in a potentially inflammatory adipose tissue macrophage phenotype. In humans, both subcutaneous (19) and visceral (33-34) adipocyte size are positively associated with an un-healthy metabolic profile including impaired glucose homeostasis and hyperlipidemia. However, adipocyte size is not correlated with systemic inflammation measured by circulating concentrations of TNFα, IL-6, and CRP (19). In addition to no association of inflammation with adipocyte size, MHO individuals have decreased macrophage infiltration into adipose tissue (34), which likely provides protection against increased systemic inflammation. Thus, the ability to expand adipose tissue may have protected Ossabaw swine from increases in systemic inflammation, adipose tissue macrophage infiltration and consequently adipose tissue inflammation.

In a lean state, Ossabaw and Yorkshire swine have similar adipocyte size despite large differences in mature body weight. Ossabaw swine have a genetic potential to accrue and store large amounts of energy in adipose tissue due to excess caloric intake; however, genetic selection of Yorkshire swine for lean mass growth limits the ability of Yorkshire swine to store energy in adipose tissue. Potentially, phenotypical and genetic differences between Ossabaw and Yorkshire swine will translate to differences in adipocyte cell culture. Despite less lipid filling by Ossabaw SVC derived adipocytes, they have increased adipogenesis as evidenced by increased clonal expansion, higher expression of PPARγ, and higher G3PDH activity compared to Yorkshire SVC derived adipocytes. Thus, we

hypothesized that Ossabaw adipocytes will be protected from palmitate-induced inflammation, and therefore insulin resistance, due to their ability to expand adipose tissue *in vivo*.

To our knowledge, this is the first study to evaluate the effect of palmitate in regards to NFκB activation and insulin resistance in porcine SVC derived adipocytes. Similar to 3T3 L1 adipocytes (2; 5; 11; 41), palmitate induced activation of NFκB in both Ossabaw and Yorkshire adipocytes. In spite of no differences in palmitate-induced NFkB activation between Ossabaw and Yorkshire adipocytes, IL-8 production was higher in Yorkshire adipocytes. Consistent with this, there were no obesity-linked increases in serum or adipose tissue concentrations of IL-8 in Ossabaw swine. IL-8 is a chemokine that increases neutrophil extravasation into tissue during the initial stages of the immune response, and may be involved in the initial stages of adipose tissue inflammation in obesity.

Neutrophil infiltration into adipose tissue happens in the early stages of high fat feeding in mice (13), although they are not detectable after 16 weeks. There is a paucity of information regarding neutrophil infiltration in human adipose tissue in obesity. Neutrophils were not detected in adipose tissue of severely obese women, but there was increased circulating myeloperoxidase compared to lean women (32), suggesting that while not detected in adipose tissue, neutrophil activation is a component of obesity. Ossabaw adipocytes are protected against palmitate-induced IL-8 production, and thus likely neutrophil infiltration into adipose tissue of obese swine. This is confirmed by the absence of myeloperoxidase activity in obese Ossabaw swine.

Inflammation due to palmitate can be induced by a number of factors other than NFκB, including JNK, ERK, and p38 MAPK (2; 11; 41). The lack of IL-6 production by

either Ossabaw or Yorkshire adipocytes in response to palmitate does not preclude the involvement of these factors in inducing IL-8 production, as palmitate induces NFκB activation without subsequent IL-6 production. Induction of IL-6 by LPS in both Ossabaw and Yorkshire adipocytes demonstrate distinct differences in Tlr activation between LPS and Palmitate. Evidence from human adipocyte cell culture suggests that the majority of inflammation is from the preadipocyte fraction (9). Thus, the degree of SVC differentiation may be a determining factor in cytokine production.

Inflammation has been linked to the development of insulin resistance. Both palmitate and LPS decreased insulin-stimulated 2-NBDG uptake by adipocytes. Perhaps most interesting is the lower basal and insulin-stimulated 2-NBDG uptake by Ossabaw adipocytes, suggesting differences in capacity to uptake glucose. Faris *et al.* (14) demonstrated that lean fasted Ossabaw swine challenged with 0.25 U/kg body weight insulin have a blood glucose concentration of 60.2 mg/dL (decrease of 57.6 mg/dL) after 15 minutes, while fasted Yorkshire swine challenged with 0.1 U/kg body weight insulin have a blood glucose concentration of 32.6 mg/dL (decrease of 46.8) after 15 minutes (Faris, R.J. and M.E. Spurlock, unpublished data). This is a substantial increase in insulin sensitivity in Yorkshire swine vs. Ossabaw swine, although differences is lean body mass may affect insulin responsiveness. Nonetheless, this increase in insulin sensitivity is reflected in adipocyte cell culture.

Taken together, this data demonstrates that Ossabaw adipocytes have increased adipogenesis, and that this may be protective against inflammation as Ossabaw adipocytes did not produce IL-8 in response to palmitate. Furthermore, Ossabaw adipocytes have decreased 2-NBDG basal vs. insulin stimulated glucose uptake which is reflective of *in vivo*

evidence of insulin sensitivity in Ossabaw and Yorkshire swine. This *in vitro* evidence supports that the marked capacity for adipose expansion in Ossabaw swine to protect against high fat diet-induced inflammation, with respect to IL-8 production, but not NFκB activation. Additionally, this data is supported by the absence of obesity-linked increases in serum and adipose tissue IL-8 concentrations.

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Figures

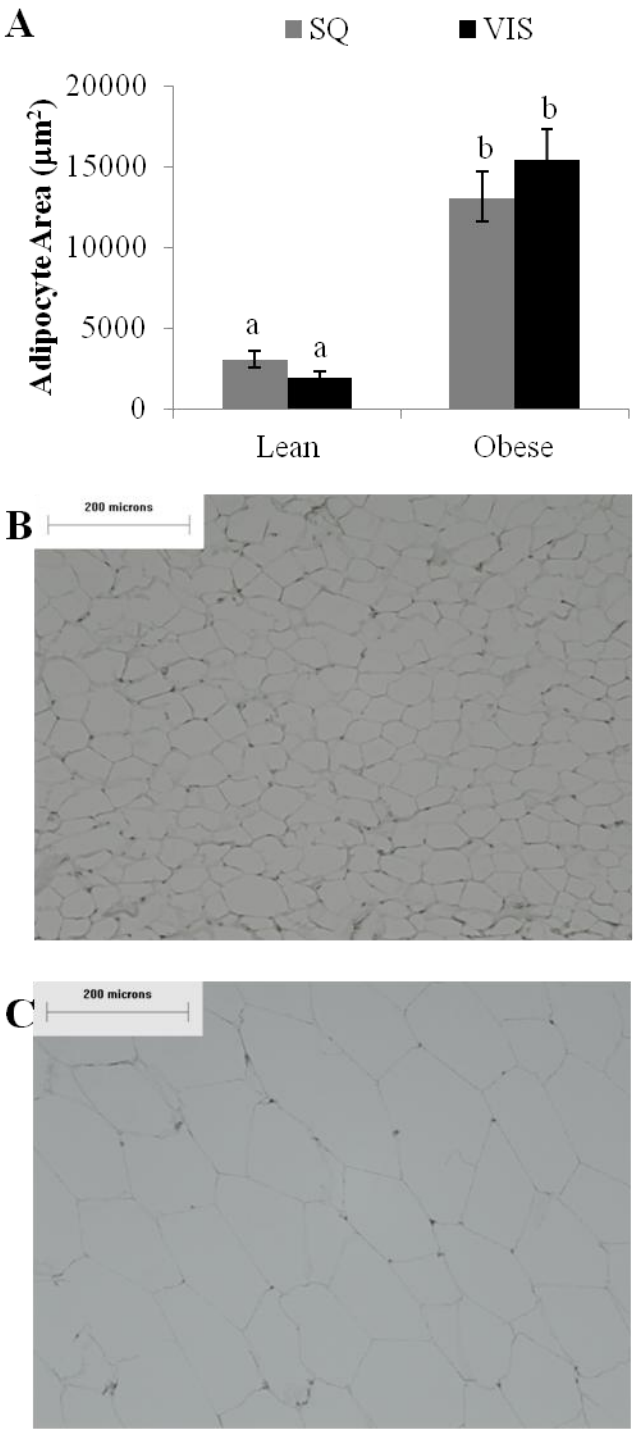

Figure 5. *Comparison of SQ and VIS adipocyte size in lean and obese Ossabaw Swine.*

(A) Adipocyte area of SQ and VIS adipose tissue sections from lean (LFC) and obese (HFP and HFPn3) Ossabaw swineand examples of (B) lean and (C) obese Ossabaw swine adipose tissue sections. Data presented is Least-Square Means ± standard error. Main Effects: (A) Diet $p<0.0001$, Tissue $P=0.31$, Diet*Tissue $p=0.0315$.

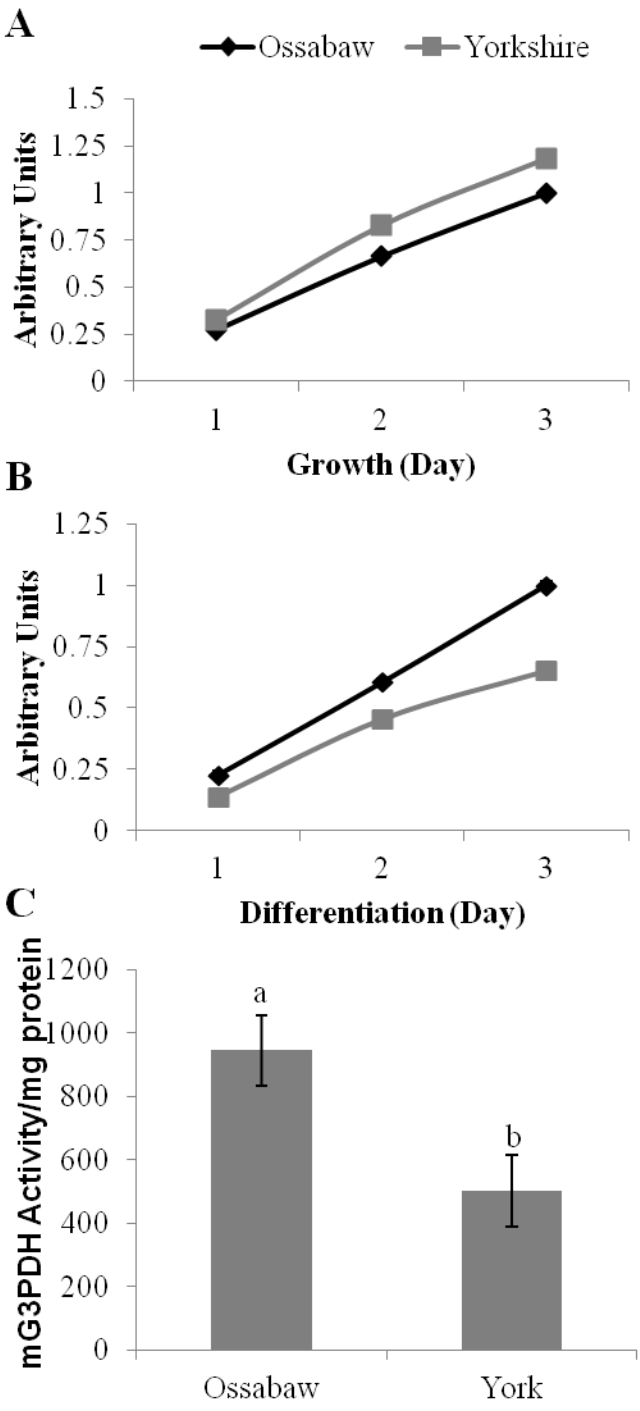

Figure 6. *Ossabaw adipocytes have increased clonal explansion and G3PDH activity compared to Yorkshire adipocytes.*

(A) BrDU incorporation during growth phase and (B) differentiation phase of Ossabaw and Yorkshire SVC. (C) G3PDH activity of Ossabaw and Yorkshire adipocytes 10 days post differentiation. Data presented is Least-Square Means ± standard error. Means with different letters represent a difference of $p<0.05$. Main Effects: (A) Genotype $p<0.0001$, Day $p<0.0001$, Genotype*Day $p=0.0048$; (B) Genotype $p<0.0001$, Day $p<0.0001$, Genotype*Day $p<0.0001$; (C) Genotype $p=0.0104$. Each experiment was replicated three times with an n=4 for each experiment.

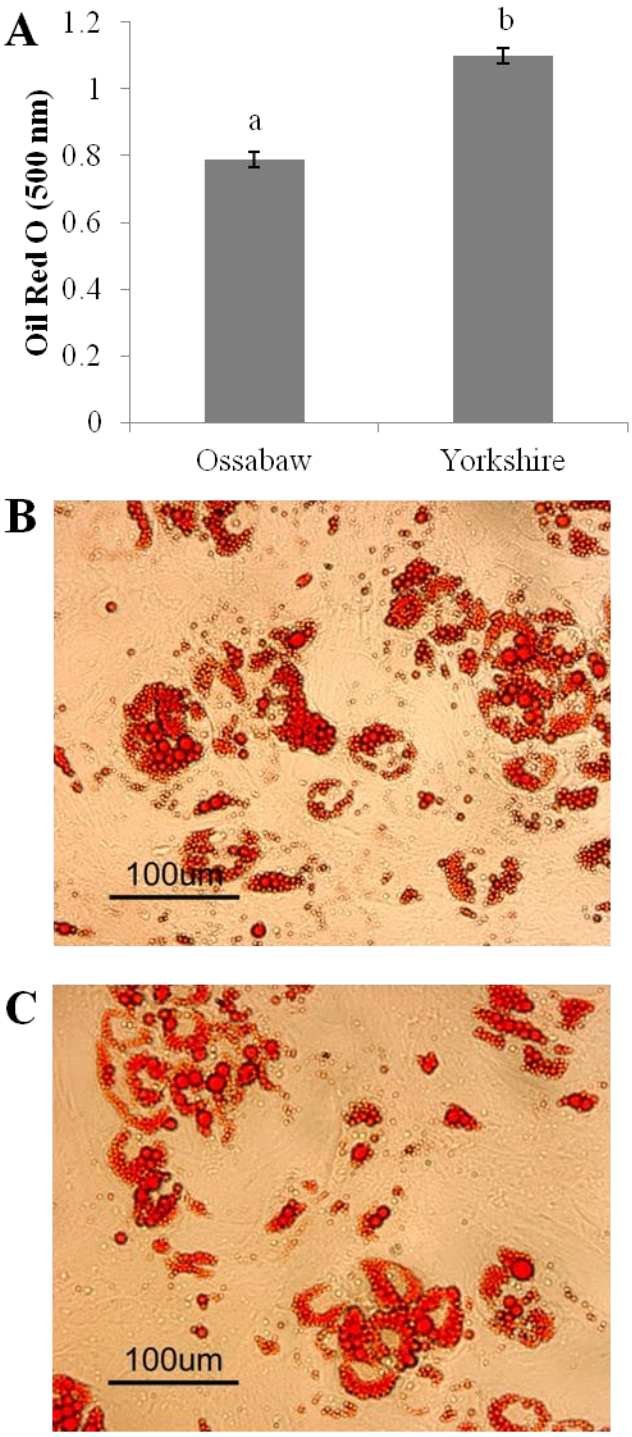

Figure 7. *Yorkshire adipocytes have more lipid filling than Ossabaw adipocytes.*

(A) Oil Red O extracted from Ossabaw and Yorkshire adipocytes 10 days post-differentiation. Representative (B) Ossabaw and (C) Yorkshire Oil Red O images. Data presented is Least-Square Means ± standard error. Means with different letters represent a difference of $p<0.05$. Main Effect: (A) Genotype $p<0.0001$. Each experiment was replicated three times with an n=6 for each experiment.

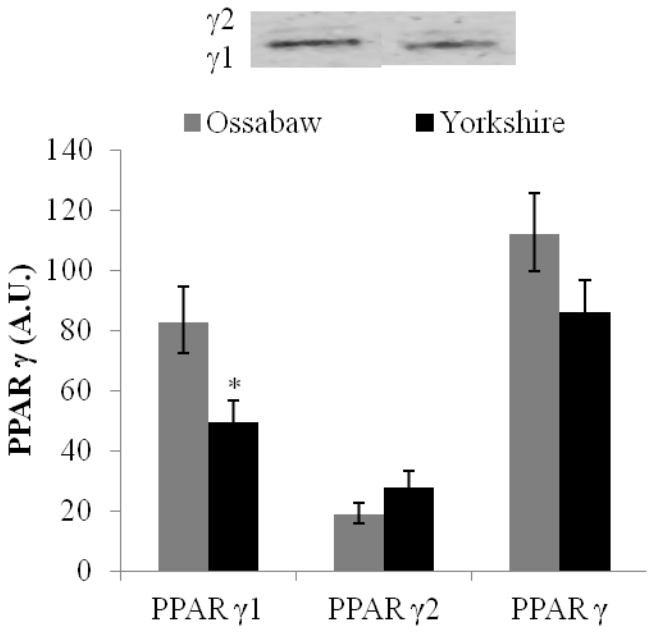

Figure 8. *Ossabaw adipocytes express more cellular PPARγ1 than Yorkshire adipocytes.*

Cellular PPARγ1, PPARγ2, total PPARγ, and representative blot in Ossabaw and Yorkshire adipocytes treated with Vehicle, Palmitate, and LPS. There was no difference by treatment. Data presented is Least-Square Means ± standard error for Ossabaw and Yorkshire adipocytes. * Represents differences ($p<0.05$) between Ossabaw and Yorkshire adipocytes Main Effects: (PPARγ1) Genotype p=0.0151, Treatment p=0.33, Genotype*Treatment p=0.40; (PPARγ2) Genotype p=0.13, Treatment p=0.51, Genotype*Treatment p=0.81; (PPARγ) Genotype p=0.13, Treatment p=0.29, Genotype*Treatment p=0.43. PPARγ assay was performed on an evenly mixed SVC lot of three pigs with an n=4.

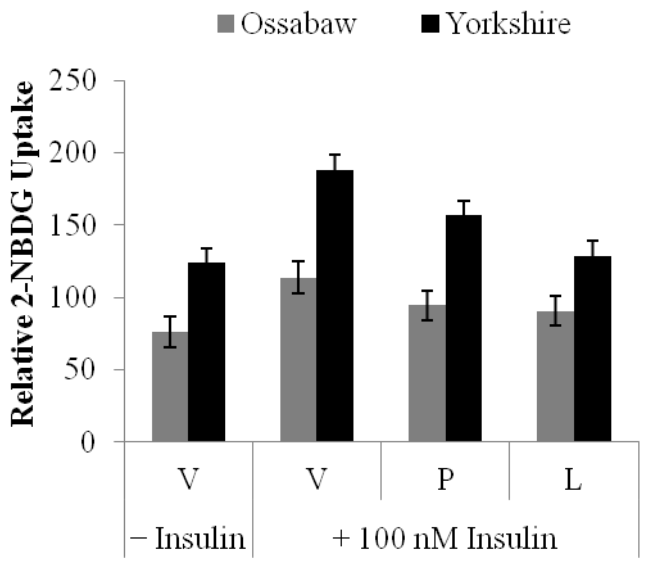

Figure 9. *Ossabaw adipocytes have decreased insulin sensitivity compared to Yorkshire adipocytes, but do not respond differently to palmitate or LPS treatment.*

Insulin stimulated 2-NBDG uptake in Ossabaw and Yorkshire adipocytes treated with Vehicle (V), Palmitate (P), and LPS (L). Data presented is Least-Square Means ± standard error. Main Effects: Treatment $p<0.0001$; Genotype $p<0.0001$, Treatment*Genotype $p=0.33$. Each experiment was replicated three times with an n=4 for each experiment.

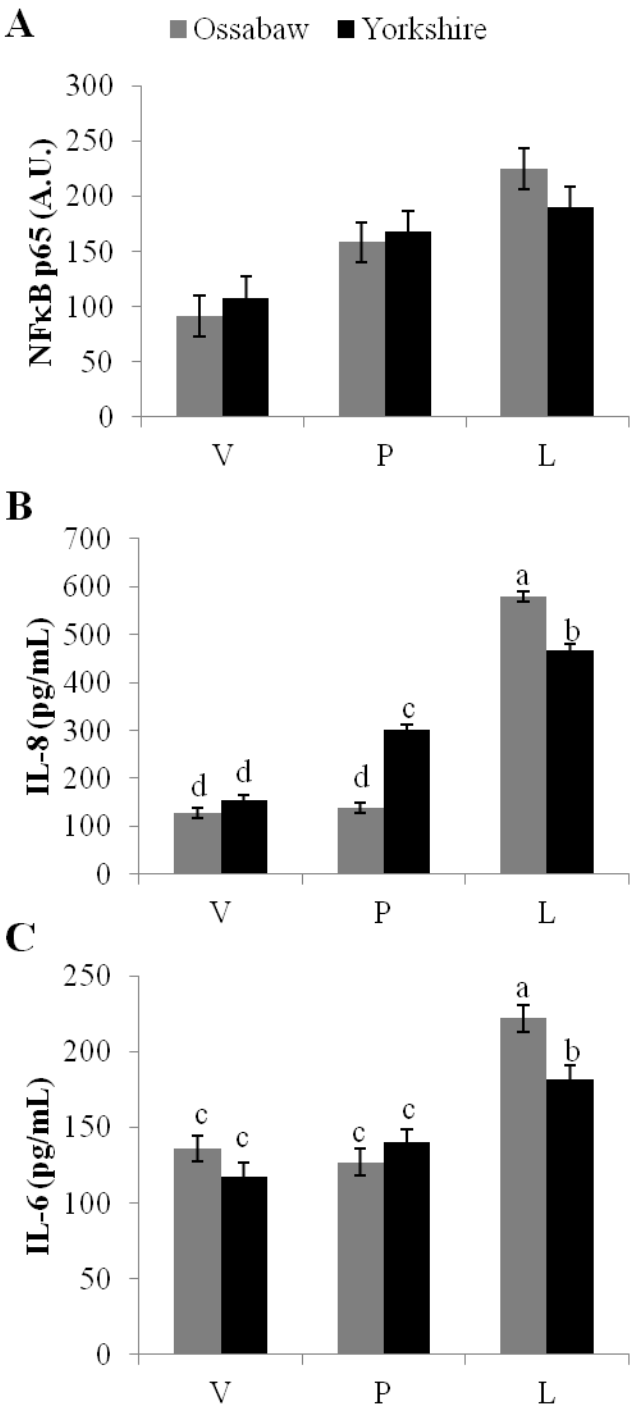

Figure 10. *Palmitate and LPS differentially regulate inflammation in Ossabaw and Yorkshire adipocytes.*

(A) NFκB p65 nuclear translocation, (B) media IL-8, and (C) media IL-6 in Ossabaw and Yorkshire adipocytes treated with Vehicle (V), Palmitate (P), and LPS (L). Data presented is Least-Square Means ± standard error. Different letter represent a difference of $p<0.05$. Main Effects: (A) Treatment $p<0.0001$, Genotype $p=0.86$, Treatment*Genotype $p=0.35$; (B) Treatment $p<0.0001$, Genotype $p=0.0037$, Treatment*Genotype $p<0.0001$; (C) Treatment $p<0.0001$, Genotype $p=0.0396$, Treatment*Genotype $p=0.0143$. (A) NFκB assay was performed on an evenly mixed SVC lot of three pigs with an n=5. (B) IL-8 and (C) IL-6 assays were replicated 3 times with an n=4 for each experiment.

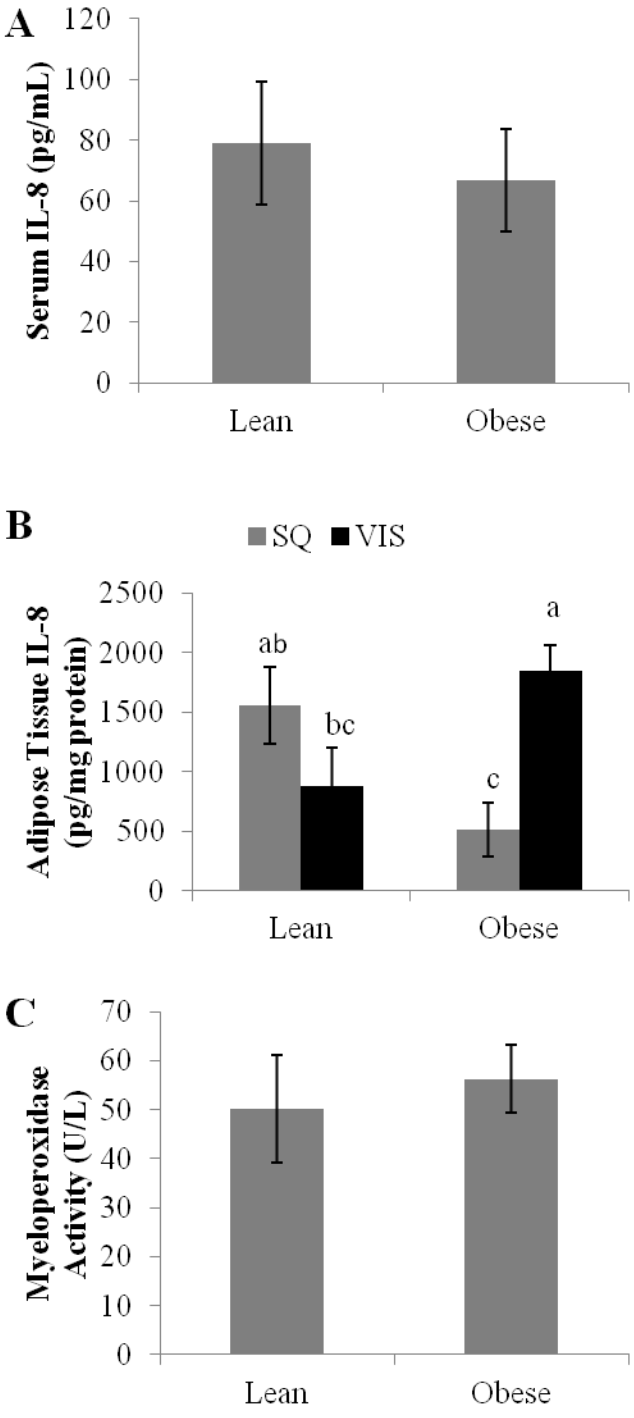

Figure 11. *Ossabaw swine do not have obesity-linked increases in IL-8 or myeloperoxidase activity.*

(A) Serum IL-8, (B) SQ and VIS adipose tissue IL-8 and (C) serum myeloperoxidase activity of lean and obese Ossabaw swine. Data presented is Least-Square Means ± standard error. Different letter represent a difference of $p<0.05$. Main Effects: (A) Diet $p=0.63$; (B) Diet $p=0.90$, Tissue $p=0.21$, Diet*Tissue $p=0.0004$; (C) Diet $p=0.65$.

CHAPTER 5: ADIPOSE TISSUE DEPOT DIFFERENCES IN MICROARRAY EXPRESSION IN OSSABAW SWINE

Abstract

Obesity is characterized by the increase in adipose tissue mass which can occur in multiple adipose tissue depots. In particular, visceral adiposity is associated with an increased risk for the development of type 2 diabetes and cardiovascular disease. We determined the global gene expression of subcutaneous and visceral adipose tissue by Affymetrix microarray analysis under conditions of restricted (n=7) or *ad libitum* (n=7) feed intake for 38 weeks in Ossabaw swine. No genes were determined to be differentially expressed by dietary treatment, however, 1494 were determined to be differentially expressed ($Q<0.05$) between subcutaneous and visceral adipose tissue, of which 777 were annotated. Gene ontology and KEGG Pathway analysis were performed using DAVID. The gene ontology cellular component category extracellular region was determined to be over-represented with 36 genes by depot. The over-represented KEGG pathways identified included arachidonic acid metabolism and chemokine signaling pathway by depot. These genes and pathways identify distinct differences in subcutaneous and visceral adipose tissue that may be important in obesity.

Introduction

Obesity, by definition, is the increase in adipose tissue mass which can occur in multiple anatomically distinct adipose tissue depots (i.e. subcutaneous, retroperitoneal, mesenteric, omental, etc.). In addition to adipose tissue depots, obesity may lead to ectopic lipid deposition in other tissues, particularly the liver (11), muscle (36), and pancreas (38). Waist circumference, which is reflective of abdominal or visceral adiposity and is a component of the metabolic syndrome (1), has been increasing in both men and women in the United States (29). Visceral adiposity increases the risk of development of the metabolic syndrome (27; 31), and thus increases the risk for development of type 2 diabetes (16) and cardiovascular disease (18; 31).

Anatomical location of the adipose tissue depot is a determinant in the functional properties of the tissue. Enzymes involved in fatty acid metabolism are differentially regulated between adipose tissue depots. Visceral adipocytes have increased acyl-Co-A synthetase (2) and diacylglycerol acyltransferase (DGAT) (2; 22) activity compared with subcutaneous adipocytes in overweight individuals, but DGAT is not increased in obese individual's visceral adipocytes (22). In addition to increased enzyme activities, visceral adipose tissue has increased inflammatory gene expression. Compared to subcutaneous adipose tissue, visceral adipose tissue has increased expression of 12/15 lipoxgenase, interleukin (IL) 6 , IL-12 p35, CXCL10 (10) , CCL2 (5), and IL-8 (4). Additionally, macrophage secreted factors increase IL-6 production more in visceral adipocytes than subcutaneous adipocytes (35). The increased inflammatory profile of visceral adipocytes is important because it can contribute directly to increased liver production of C-reactive protein (CRP) (3), and further the chronic low-grade inflammation associated with obesity.

The need for alternative comparative models to investigate differences in adipose depot gene expression under lean and obese conditions is evident. Murine models have been pivotal to investigating the etiology of obesity and the roles of specific genes. However, human and mice differ on a number of factors relating to studying adipose tissue and obesity. Specifically, CRP is not highly expressed nor is it an acute phase protein in mice (39), human macrophages do not produce nitric oxide (32), and gonadal fat is not a major adipose tissue depot in humans. Swine are a viable alternative due to their comparative physiology and anatomy. Ossabaw swine are particular interest because of their ability to accumulate fat mass and their previous use in studying obesity and its co-morbidities (12; 15; 28). Thus, the objective of this research was to determine global gene expression in subcutaneous and visceral adipose tissue in lean and obese Ossabaw swine.

Methods

Animals. All animal procedures were conducted at Iowa State University and were approved by the Iowa State University Institutional Animal Care and Use Committee. Fourteen sexually mature male and female Ossabaw swine (*Sus scrofa*) (average initial body weight 46.7 kg) were housed in individual pens in a temperature controlled environment with a 12 hour light cycle. Swine were obtained from the Comparative Medicine Program at the Indiana University School of Medicine and Purdue University from a herd that tested negative for *Brucella* spp. and psuedorabies, and showed no clinical evidence of communicable disease upon arrival or during the study. Swine were fed a diet formulated to meet minimum requirements for mature swine (33) for 38 weeks. Dietary treatments were *ad libitum* feed (n=7; 4 male, 3 female) or restricted feed (n=7, 3 male, 4 female). After 38 weeks of dietary treatment, animals were fasted 12 hours before blood collection via jugular

venipuncture. Thereafter, animals were anesthetized with telazol:ketamine:xylazine (1:1:1) and then were euthanized by exsanguination. Subcutaneous (SQ) adipose tissue from back fat above the 10^{th} rib and visceral adipose tissue (VIS) surrounding the intestines were collected and snap frozen in liquid nitrogen.

Serum analysis. Cholesterol, glucose, triglycerides, and non-esterified fatty acids (NEFA) were analyzed using Chol Slides (Ortho Clinical Diagnostics, Rochester, NY), Glu slides (Ortho Clinical Diagnostics), Trig Slides (Ortho Clinical Diagnostics), and NEFA-HR (Wako Diagnostics, Richmond, VA), respectively, on a Vitros 5.1 Chemistry Analyzer (Ortho Clinical Diagnostics). Serum insulin concentrations were measured by EIA (ALPCO, Salem, NH). The HOMA-IR was calculated using fasting serum insulin (μU/mL) x fasting blood glucose (mg/dL) divided by 405 (30). Serum endotoxin was determined using Chromogenic LAL Assay (Lonza, Anaheim, CA).

RNA Isolation. RNA was isolated from SQ and VIS adipose tissue by Guanidinium Thiocyanate-Phenol-Chloroform extraction as previously described (7). Quality and quantity of RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Microarray analysis was performed at the Iowa State University GeneChip Facility (Ames, IA) using Affymetrix Porcine GeneChip Genome Array according to manufacturer's directions (Affymetrix, Santa Clara, CA).

Microarray analysis. Transcript levels were normalized to the chip median and log transformed. For each probeset, the generalized linear model $Y_{ij} = \mu + B_1 T_i + \varepsilon_{ij}$ was fit. In each ANOVA, Y_{ij} is a the log normalized transcript level for the i^{th} treatment and the j^{th} replicate, μ is the overall mean expression for the feature and T_i represents the i^{th} treatment (Treatment, Depot, Sex, Treatment*Depot, Treatment*Sex, Depot*Sex,

Treatment*Depot*Sex). An F test of the effect of treatment for each probeset was conducted as the ratio of the mean squares for treatment over the mean squares for error, and the p value for the test of the null hypothesis (i.e., mean expression not different among the treatments) was calculated. We examined the model for conformation to the assumption of normality of the residuals testing the null hypothesis that the residuals for each gene were normally distributed using the Shapiro-Wilkes Test. For each p-value, a Q-value was determined to control for FDR. All analyses were performed in SAS 9.2 (SAS Institute, Cary, NC). If the test of the null hypothesis of difference across treatments was rejected, and we had no evidence for departure from normality of the residuals, we declared the gene differentially expressed across treatments. Microarray data was annotated with gene title, gene symbol, and Entrez gene ID (9) (Hu, Z., J. Reecy, and C. Tuggle. Unpublished data). DAVID (23-24) was used to determine over-represented (EASE Score $p<0.05$) biological process, cellular component, and molecular functional categories using the GO FAT databases. Additionally, over-represented KEGG pathways were also determined.

Statisical analysis. Statistical procedures for serum analysis was performed using SAS 9.2 (SAS, Cary, NC). Residuals were analyzed to detect outliers and normality using the PROC Univariate procedure. Right-skewed data were log-transformed for further analysis. ANOVA was performed using the PROC MIXED procedure. The main effect was dietary treatment. Sex was considered a fixed effect and was included in the model when $p\leq0.15$ for the fixed effect or interaction with dietary treatment.

Results

After 38 weeks of dietary treatment *ad libitum* fed Ossabaw swine became obese weighing an average of 118.8 kg compared to the restricted fed Ossabaw swine weighing an

average of 59.8 kg. At the end of the study, obese Ossabaw swine were metabolically healthy (**Table 5**), with was no differences in circulating serum concentrations of insulin, glucose, NEFA, or endotoxin. Nor was there a difference in HOMA-IR. Both serum cholesterol and triglycerides were increased by dietary treatment, although the increase likely was not pathological.

To determine the effect of dietary treatment, adipose tissue depot, sex, and their interactions, microarray analysis was performed on SQ and VIS adipose tissue. At total of 19,540 genes were determined to be expressed and included in further analysis. Of these, a total of 9617 genes were successfully annotated. After controlling for FDR, only 7 genes by sex and 1494 genes by depot were determined differentially expressed ($Q<0.05$). Only one gene (thymosin beta 4, X linked) out of 7 was annotated for sex effect. For the depot effect, 777 out of 1494 differentially expressed genes ($Q<0.05$) were annotated. At a cutoff of $Q<0.01$, 95 out of 185 genes were annotated. This data is presented in **Table 6**, with the Affy ID, gene title, Q-value, and fold change. Of these genes, a total of 38 genes were up-regulated in VIS adipose tissue, relative to SQ adipose tissue, while 57 were down regulated.

To determine over-represented gene categories and pathways, the 777 annotated differentially expressed genes ($Q<0.05$) were analyzed by DAVID using the Entrez Gene ID for gene ontology molecular function, cellular component, and biological process categories (GOFAT) and the KEGG pathway database. The Entrez Gene IDs for all annotated genes were used as the background list. In all, only one cellular component (extracellular region) category and two KEGG pathways (arachidonic acid metabolism and chemokine signaling pathway) were determined to be over-represented (EASE Score<0.05) by differently expressed genes by depot. Genes expressed in extracellular region, arachidonic acid

metabolism, and chemokine signaling pathway categories are listed in **Tables 7-9**, respectively, and will be discussed further in the next section.

Discussion

Despite the duration of the study and body weight gain by the *ad libitum* fed Ossabaw swine, they did not display a perturbed metabolic profile. Nor did they have any differentially expressed genes by dietary treatment after controlling for FDR. Dietary factors may be casual to the development of an un-healthy metabolic profile, and the absence of high dietary fat may have precluded the development of altered glucose homeostasis, hyperlipidemia, and altered adipose tissue gene expression. As evidenced by others (12; 28), the inclusion of high dietary fat and supra-physiological levels of cholesterol induces hyperlipidemia in Ossabaw swine. Additionally, high dietary fat in itself is enough to induce insulin resistance in Ossabaw swine (15). Nonetheless, distinct differences between SQ and VIS depots were evident by microarray analysis.

The gene ontology cellular component extracellular region refers to gene products that are secreted outside the cell. Thirty-six genes were over-represented in this category (**Table 7)** from the significant differentially expressed genes by depot. Four of these genes are involved in the insulin-like growth factor (IGF) axis: IGF1, IGFBP3, IGFBP5, and IGFBP6. There is a relative paucity of information relating to IGF-1 and its binding protein in regards to different adipose tissue depots. IGF-1 is important in both the proliferation of preadipocytes and differentiation into adipocytes (20; 37). However, IGF-1-induced PKB signaling is impaired in visceral adipocytes compared to subcutaneous adipocytes (8). IGF-1 and IGFBP-3 are decreased in response to increased body fat, especially in women (19). Additionally, increasing IGF-1 concentrations via growth hormone supplementation

decreases visceral adiposity (17; 21; 34). Whether this is because of increasing IGF-1 or directly to growth hormone is unclear. Potentially, lower concentrations of IGF-1 and IGFBP in visceral adipose tissue result in decreased proliferation and cell survival and thus, contribute to adipose tissue dysfunction.

In addition to IGF proteins, three chemokines were also identified as over-represented genes in the extracellular region gene ontology cellular component category (**Table 7**). These chemokines are CCL2, CXCL2, and CCL21. CCL2 and CXCL2 were down-regulated in visceral adipose tissue compared to subcutaneous whereas CCL21 was up-regulated. Chemokine signaling pathway was also indentified as a KEGG pathway containing over-represented genes (**Table 9**), including CCL2, CXCL2, CCL21, and CCR3. CCL2 is increased in obesity (5), and its importance in macrophage infiltration into adipose tissue is well established (26). In humans, CCL2 is produced at a higher rate in visceral than subcutaneous adipose tissue, however, after adjusting for tissue macrophages this difference disappeared (5). Based upon the lack of metabolic and microarray differences by dietary treatment, it is likely that the decreased CCL2 expression in visceral adipose tissue is due to lack of pathological consequences from diet induced obesity and only reflects strictly depot differences. Other than increased expression of CCR3 in subcutaneous adipose tissue in obesity (25), literature pertaining to CCR3, CXCL2, and CCL21 pertaining to obesity and adipose tissue depots is absent.

In addition to chemokine signaling pathway, seven genes were identified in the arachidonic acid metabolism KEGG Pathway as containing over-represented genes by depot in our microarray analysis (**Table 8**). Products of arachidonic acid metabolism by 12/15 lipoxygenase induce inflammation and insulin resistance in 3T3 L1 adipocytes (6). Like

humans, our data demonstrates that arachidonate 12/15 lipoxygenase is more highly expressed in visceral than subcutaneous adipose tissue (10). Literature relating to obesity and adipose tissue is limited for the other gene indicated in arachidonic acid metabolism KEGG pathway. One gene, prostaglandin G/H synthase-2 (i.e. COX-2) has been implicated in obesity in rodent models (13). In humans, COX-2 expression is higher in epicardial adipose tissue than subcutaneous, but lower than that of visceral adipose tissue (14).

While global gene expression analysis did not identify genes differentially regulated by dietary treatment, it did identify differences in mRNA abundance of genes related to excreted proteins, arachidonic acid metabolism, and chemokine signaling pathway between SQ and VIS adipose tissue. These genes may be important with respect to obesity and adipose tissue inflammation and dysfunction. Further investigation into these genes in VIS and SQ adipose tissue with a high fat dietary treatment is warranted.

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Tables

Table 5. *Metabolic profile of Ossabaw swine.*

Parameter	Restricted	Obese	p value
Insulin (pg/mL)	119.7±25.8	130.1±25.8	0.78
Glucose (mg/dL	93.6±16.2	106.9±16.2	0.57
HOMA-IR	0.57±0.15	0.75±0.15	0.42
Cholesterol (mg/dL)	63.4±5.6	81.0±5.6	0.0463
Triglycerides (mg/dL)	21.6±2.1	37.5±2.1	0.0005
NEFA (mM)	0.28±0.06	0.43±0.06	0.13
Endotoxin (ng/mL)	14.9±1.3	14.7±1.3	0.91

Table 6. *Differentially expressed genes by depot (Q<0.01).*

Affy ID	Rank[a]	GENE TITLE	Q-value	Fold Change[b]
Ssc.24864.1.A1_at	1	hypothetical LOC100515948	0.0007	13.40
Ssc.8623.1.A1_at	4	Sec23 homolog A (S. cerevisiae)	0.0014	-5.08
Ssc.5713.1.S1_at	7	gelatinase A	0.0015	-50.51
Ssc.3450.1.S1_at	8	homeobox protein Hox-D8-like	0.0029	-4.87
Ssc.12936.1.A1_at	10	keratocan-like	0.0033	-5.70
Ssc.18608.1.S1_at	15	cellular retinoic acid binding protein 1	0.0039	1.60
Ssc.15923.1.S1_at	18	fibroblast growth factor 7	0.0039	-0.25
Ssc.25680.1.S1_at	19	mutS homolog 3 (E. coli)	0.0040	0.20
Ssc.21925.1.S1_at	22	complement C1q tumor necrosis factor-related protein 5-like	0.0040	-3.53
Ssc.2445.1.S1_at	23	microfibrillar-associated protein 2-like	0.0040	-5.72
Ssc.2464.1.S1_at	25	Stanniocalcin 1	0.0040	-4.30
Ssc.21549.1.A1_at	28	meningioma expressed antigen 5 (hyaluronidase)	0.0040	0.89
Ssc.15266.1.S1_at	32	15-hydroxyprostaglandin dehydrogenase [NAD+]-like	0.0042	1.80
Ssc.6327.1.S1_at	33	keratin, type I cytoskeletal 19-like	0.0042	5.95
Ssc.21125.1.S1_at	35	oral-facial-digital syndrome 1	0.0045	-1.02
Ssc.30175.1.A1_at	36	carboxypeptidase X (M14 family), member 1	0.0045	-8.90
Ssc.16418.2.S1_at	37[c]	integrin beta-like protein 1-like	0.0052	-3.94
Ssc.16589.1.S1_at	41	collagen, type VI, alpha 3	0.0053	-48.52
Ssc.8573.1.A1_at	43	apolipoprotein D	0.0053	-40.40
Ssc.30.1.S1_at	46	proteasome (prosome, macropain) subunit, alpha type, 4	0.0053	-7.89
Ssc.19688.1.S1_at	47	cholinergic receptor, nicotinic, alpha 1 (muscle)	0.0053	-23.30
Ssc.12795.1.S1_at	48	three prime repair exonuclease 1-like	0.0055	-1.10
Ssc.24330.1.S1_at	49	cytochrome P450, family 7, subfamily B, polypeptide 1	0.0056	2.89
Ssc.4044.1.A1_at	52	hypothetical LOC100512560	0.0059	2.62
Ssc.26893.1.A1_at	53	hemicentin-1-like	0.0059	2.64
Ssc.26617.1.A1_at	54	paired mesoderm homeobox protein 1-like	0.0059	-5.25

Table 6. *Differentially expressed genes by depot (Q<0.01) (continued).*

Ssc.5396.1.S1_at	59	Similar to transcription factor 21	0.0062	3.13
Ssc.24864.2.S1_at	60	hypothetical LOC100515948	0.0062	2.08
Ssc.9754.1.A1_at	62	Ankyrin repeat and SOCS box-containing 6	0.0064	-0.44
Ssc.24250.1.S1_at	63	cellular retinoic acid binding protein 2	0.0065	-11.22
Ssc.18278.2.A1_at	66	nebulin-related anchoring protein	0.0067	-0.45
Ssc.6058.1.S1_at	67	dual specificity phosphatase 1	0.0067	-34.67
Ssc.6817.1.A1_a_at	73	tumor suppressor candidate 3-like	0.0068	1.93
Ssc.2747.1.S1_at	74	homeobox protein Hox-B5-like	0.0068	1.74
Ssc.1600.3.S1_at	78	ribosomal protein S6 modification-like protein B-like	0.0068	1.21
Ssc.226.1.S1_at	83	high-mobility group box 2	0.0068	1.61
Ssc.26825.1.S1_at	85	zinc finger protein 330-like	0.0068	1.00
Ssc.14851.1.S1_at	86	amyloid beta (A4) precursor-like protein 2	0.0068	0.87
Ssc.11952.1.S1_at	87[c]	transmembrane protein 117-like	0.0068	-1.23
Ssc.17345.1.S1_at	88	angiopoietin-like 4	0.0068	-2.56
Ssc.19323.1.S1_at	89	protein NOXP20-like	0.0070	-6.46
Ssc.24429.1.A1_at	90	sperm-specific antigen 2-like	0.0070	-0.20
Ssc.1310.1.S1_at	91	prostaglandin E synthase	0.0070	-4.09
Ssc.19136.1.S1_at	93	latent-transforming growth factor beta-binding protein 2-like	0.0071	1.73
Ssc.11369.2.A1_at	97	Der1-like domain family, member 1	0.0071	-2.80
Ssc.8027.1.A1_at	98	dickkopf homolog 3 (Xenopus laevis)	0.0071	5.16
Ssc.5895.1.A1_at	99	collagen, type VI, alpha 1	0.0071	-6.72
Ssc.10793.1.A1_at	100	syndecan 2	0.0071	-10.35
Ssc.20706.1.S1_at	102	hypothetical protein LOC100523635	0.0071	2.94
Ssc.4048.1.A1_at	107	neogenin-like	0.0074	2.43
Ssc.26172.1.S1_at	108	leprecan-like 4	0.0074	-1.01
Ssc.4381.1.S1_at	111	septin 11	0.0077	-8.88
Ssc.820.1.S1_at	113	alanyl (membrane) aminopeptidase	0.0079	-11.29
Ssc.1986.1.S1_at	114	Prostaglandin-endoperoxide synthase 1	0.0079	2.31
Ssc.2425.1.S1_at	115	zonula occludens 1	0.0079	5.21

Table 6. *Differentially expressed genes by depot (Q<0.01) (continued).*

Ssc.11185.1.A1_at	117	SH3 domain-containing kinase-binding protein 1-like	0.0079	-1.01
Ssc.15233.1.S1_at	119	Similar to Homeobox protein SIX1 (Sine oculis homeobox homolog 1)	0.0079	-11.80
Ssc.14134.1.S1_at	120	polypeptide N-acetylgalactosaminyltransferase 1-like	0.0079	-2.70
Ssc.28359.1.A1_at	121	hypothetical protein LOC100153390	0.0079	-0.72
Ssc.7769.2.S1_at	122	lysophosphatidylglycerol acyltransferase 1	0.0080	-7.40
Ssc.1495.1.S1_a_at	125	transcription elongation factor A protein 1-like	0.0081	2.60
Ssc.12446.1.A1_at	127	caspase-13-like	0.0081	-2.16
Ssc.21869.1.S1_at	128	src kinase-associated phosphoprotein 2-like	0.0081	3.60
Ssc.1091.2.S1_at	130	collagen, type I, alpha 1	0.0081	-5.53
Ssc.5895.1.A2_at	132	Collagen, type VI, alpha 1	0.0081	-12.83
Ssc.25217.1.S1_a_at	133	selenoprotein M	0.0081	-6.89
Ssc.11628.1.A1_at	135	GPN-loop GTPase 1-like	0.0081	-1.14
Ssc.15646.1.S1_at	136	lipase maturation factor 2-like	0.0081	-1.66
Ssc.373.1.S1_at	137	matrix Gla protein	0.0081	35.16
Ssc.8072.2.A1_at	139	latent-transforming growth factor beta-binding protein 1-like	0.0081	3.49
Ssc.18454.1.A1_at	143	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	0.0081	2.32
Ssc.3919.1.S1_at	144	cytosolic 5'-nucleotidase III-like protein-like	0.0081	-3.73
Ssc.19566.1.S1_at	147	secreted frizzled-related protein 4	0.0081	-28.97
Ssc.27415.1.S1_at	148	homeobox protein BarH-like 1-like	0.0082	2.65
Ssc.12068.1.A1_at	149	Collagen, type VI, alpha 3	0.0082	-2.95
Ssc.19119.1.A1_at	151	homeobox protein EMX2-like	0.0082	-1.00
Ssc.5645.1.A1_at	152	lamina-associated polypeptide 2, isoforms beta/gamma-like	0.0084	1.58
Ssc.3401.1.S1_at	153	eukaryotic translation initiation factor 3, subunit M	0.0084	2.94
Ssc.11302.1.S2_at	155	hypothetical protein LOC100152001	0.0084	-67.71

Table 6. *Differentially expressed genes by depot (Q<0.01) (continued).*

Ssc.4563.1.S1_a_at	158	N-acetyltransferase 10 (GCN5-related)	0.0086	-0.34
Ssc.6105.1.A1_at	161	plexin B1	0.0087	0.90
Ssc.8000.1.A1_at	166	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	0.0091	2.69
Ssc.6736.1.S1_at	168	homeobox protein MSX-2-like	0.0094	-2.07
Ssc.24393.1.S1_at	170	reticulocalbin-3-like	0.0095	-11.50
Ssc.9061.1.A1_at	171	cystatin-M-like	0.0096	2.41
Ssc.3832.1.S1_at	172	aquaporin 3 (Gill blood group)	0.0096	-40.64
Ssc.17281.1.A1_at	174	F-box and leucine-rich repeat protein 5	0.0096	-6.54
Ssc.25178.1.S1_a_at	175	protein transport protein Sec24D-like	0.0096	-2.48
Ssc.8564.1.A1_at	178	Similar to very low density lipoprotein receptor VLDL-R2	0.0096	7.06
Ssc.22738.1.A1_at	179	protein MON2 homolog	0.0096	-1.49
Ssc.2589.1.S1_at	180	troponin I type 3 (cardiac)	0.0096	2.36
Ssc.4993.1.A1_at	181	hypothetical protein LOC100517021	0.0096	-19.82
Ssc.272.1.S1_a_at	182	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	0.0096	1.96
Ssc.26447.1.S1_at	183	carboxypeptidase D	0.0099	1.09
Ssc.18928.1.A1_at	185	Mucosal vascular addressin cell adhesion molecule 1	0.0099	1.37

[a]Ranking of all differentially expressed (Q<0.01) genes.

[b]Fold change in mRNA abundance for visceral adipose tissue relative to subcutaneous adipose tissue. A positive fold change indicates increased mRNA abundance in visceral adipose tissue relative to subcutaneous adipose tissue and a negative fold change indicates decreased mRNA adundance.

cResiduals of gene showed evidence of a non-normal distribution

Table 7. *Gene ontology cellular component: extracellular region gene list*[a].

Affy ID	Gene Title	Q-value	Fold Change
Ssc.12365.1.A1_at	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.0233	-6.48
Ssc.11257.1.S1_at	TIMP metallopeptidase inhibitor 2	0.0130	-34.61
Ssc.6009.1.S1_at	amylase, alpha 2B (pancreatic)	0.0440	-0.58
Ssc.17345.1.S1_at	angiopoietin-like 4	0.0068	-2.56
Ssc.14474.1.S1_at	Apomucin	0.0206	0.09
Ssc.16624.1.S1_at	carbonic anhydrase-related XI protein	0.0342	0.58
Ssc.1020.1.S1_at	cathepsin K	0.0170	-28.91
Ssc.657.1.A1_at	chemokine (C-C motif) ligand 2	0.0327	-4.05
Ssc.18613.1.S1_at	chemokine (C-C motif) ligand 21	0.0180	1.94
Ssc.4871.1.S1_at	chemokine (C-X-C motif) ligand 2	0.0215	-2.08
Ssc.31199.1.S1_at	collagen, type V, alpha 1	0.0179	-0.80
Ssc.61.1.S1_at	complement component 3	0.0345	31.49
Ssc.10245.2.A1_a_at	Decorin	0.0281	-41.25
Ssc.8027.1.A1_at	dickkopf homolog 3	0.0071	5.16
Ssc.16236.1.S1_at	dipeptidyl-peptidase 4	0.0489	0.67
Ssc.15923.1.S1_at	fibroblast growth factor 7 (keratinocyte growth factor)	0.0039	-0.25
Ssc.137.1.S1_at	Ficolin	0.0184	-14.97
Ssc.5833.1.A2_at	follicle stimulating hormone, beta polypeptide	0.0222	0.05
Ssc.4747.1.S1_at	Follistatin	0.0168	0.63
Ssc.5713.1.S1_at	gelatinase A	0.0015	-50.51
Ssc.14246.1.S1_at	Gelsolin	0.0302	-23.34
Ssc.16231.1.S1_a_at	insulin-like growth factor 1	0.0119	-2.68
Ssc.15588.1.S2_at	insulin-like growth factor binding protein 3	0.0339	-16.60
Ssc.15800.1.S1_at	insulin-like growth factor binding protein 5	0.0302	-10.14
Ssc.14062.2.S1_a_at	insulin-like growth factor binding protein 6	0.0281	-20.02
Ssc.62.2.S1_a_at	interleukin 6 (interferon, beta 2)	0.0470	-0.47
Ssc.373.1.S1_at	matrix Gla protein	0.0081	35.16
Ssc.734.1.S1_at	matrix metallopeptidase 14 (membrane-inserted)	0.0418	-2.07
Ssc.11194.1.S1_at	plasminogen activator	0.0383	-2.24
Ssc.196.1.S1_at	plasminogen activator, tissue	0.0296	-6.52

Table 7. *Gene ontology cellular component: extracellular region gene list*[a] *(Continued).*

Ssc.16228.1.S1_at	platelet basic protein	0.0417	1.73
Ssc.10510.1.A1_at	Prolactin	0.0480	-0.12
Ssc.14556.1.S1_at	Proopiomelanocortin	0.0136	0.10
Ssc.15695.1.S1_at	retinol binding protein 4, plasma	0.0206	19.02
Ssc.2464.1.S1_at	stanniocalcin 1	0.0040	-4.30
Ssc.5614.1.S1_at	Tryptase	0.0413	0.49

[a]Gene list of over-represented family of genes (EASE Score=0.0050) that were differentially expressed (Q<0.05).

[b]Fold change in mRNA abundance for visceral adipose tissue relative to subcutaneous adipose tissue. A positive fold change indicates increased mRNA abundance in visceral adipose tissue relative to subcutaneous adipose tissue and a negative fold change indicates decreased mRNA abundance.

Table 8. *KEGG pathway: arachidonic acid metabolism gene list*[a].

Affy ID	Gene Title	Q-value	Fold Change
Ssc.10974.1.S1_at	arachidonate 12/15-lipoxygenase	0.0124	13.64
Ssc.732.1.S1_at	carbonyl reductase 2	0.0461	3.06
Ssc.11267.1.S1_at	cytochrome P450 2B22	0.0424	2.95
Ssc.1310.1.S1_at	microsomal prostaglandin E synthase-1	0.0070	-4.09
Ssc.7314.1.A1_at	prostaglandin G/H synthase-2	0.0195	-0.12
Ssc.1986.1.S1_at	prostaglandin-endoperoxide synthase 1	0.0079	2.31
Ssc.8278.1.S1_at	soluble epoxide hydrolase	0.0264	-4.27

[a]Gene list of over-represented family of genes (EASE Score=0.0152) that were differentially expressed (Q<0.05).

[b]Fold change in mRNA abundance for visceral adipose tissue relative to subcutaneous adipose tissue. A positive fold change indicates increased mRNA abundance in visceral adipose tissue relative to subcutaneous adipose tissue and a negative fold change indicates decreased mRNA abundance.

Table 9. *KEGG pathway: chemokine signaling pathway gene list*[a].

Affy ID	Gene Title	Q-value	Fold Change[b]
Ssc.657.1.A1_at	chemokine (C-C motif) ligand 2	0.0327	-4.04524
Ssc.18613.1.S1_at	chemokine (C-C motif) ligand 21	0.018	1.938086
Ssc.19054.1.S1_a_at	chemokine (C-C motif) receptor 3	0.0488	0.169559
Ssc.4871.1.S1_at	chemokine (C-X-C motif) ligand 2	0.0215	-2.07684
Ssc.14176.1.A1_s_at	glycogen synthase kinase 3 beta	0.015	-1.22895
Ssc.16228.1.S1_at	platelet basic protein	0.0417	1.727623
Ssc.6025.1.S1_at	signal transducer and activator of transcription 1	0.0311	1.367214
Ssc.10911.2.S1_at	similar to Beta-adrenergic receptor kinase 2 (Beta-ARK-2) (G-protein-coupled receptor kinase 3)	0.0143	-3.14621
Ssc.2140.1.S1_at	similar to PTK2B protein tyrosine kinase 2 beta	0.0471	0.422968
Ssc.5093.1.A1_at	similar to SHC-transforming protein 1 (SH2 domain protein C1) (Src homology 2 domain-containing-transforming protein C1)	0.0407	-3.14165

[a]Gene list of over-represented family of genes (EASE Score=0.0152) that were differentially expressed ($Q<0.05$).

[b]Fold change in mRNA abundance for visceral adipose tissue relative to subcutaneous adipose tissue. A positive fold change indicates increased mRNA abundance in visceral adipose tissue relative to subcutaneous adipose tissue and a negative fold change indicates decreased mRNA abundance.

CHAPTER 6. GENERAL CONCLUSIONS

The prevalence of obesity in the United States is continuing to rise (2-3) and places a significant burden on our health care system (11). Research has identified that insulin resistance in obesity is related to adipose tissue inflammation (4) and that this is accompanied by macrophage infiltration into adipose tissue (12-13). Rodent models have been pivotal to obesity and obesity-related research and the role of individual genes. However, distinct differences between rodents and humans underscore the need for alternative comparative models. Unlike humans, CRP is not an acute phase protein nor is it highly expressed in mice (10). Additionally, mice macrophages are functionally different in that they produce NO via inducible nitric oxide synthase (8). Swine are an attractive alternative model due to their comparative physiology and anatomy to humans. In particular, Ossabaw swine are a suitable alternative as they can consistently develop multiple metabolic syndrome risk factors (9).

I first sought to characterize obesity-linked adipose tissue inflammation and macrophage phenotype in Ossabaw swine (Chapter 3). Therefore, I feed Ossabaw swine for 30 weeks with a restricted intake low-fat control diet, a high palm oil diet, or a high palm oil with n-3 fatty acids diet. We determined that the major adipose tissue macrophage phenotype in Ossabaw swine is $CD16^{+}CD14^{-}$, which is likely anti-inflammatory. High dietary palm oil did cause an increase in adipose tissue $CD16^{-}CD14^{+}$ macrophages, which a likely inflammatory, and this increase was attenuated by the addition of dietary n-3 fatty acids. Concomitant with a largely anti-inflammatory adipose tissue macrophage phenotype, there was no induction of inflammation measured by serum CRP, TNFα, IL-6, or IL-12. Serum adiponectin was decreased by the high palm oil diet, but this decrease was attenuated by the addition of n-3 fatty acids. However, the increase in serum adiponectin by the

addition of n-3 fatty acids did not attenuate the decrease in longissimus dorsi AMPKα phosphorylation. Some individuals remain healthy despite obesity, and possess a favorable inflammation profile (5). The ability to expand adipose tissue may afford protection against adipose tissue expansion. As evidenced by Kim *et al.* (6), adipose tissue expansion attenuates inflammation due to obesity.

However, the metabolic profile of obese Ossabaw swine in this study was not entirely healthy. There was no increase in serum cholesterol or triglycerides, but there was apparent hyperglycemia without hyperinsulinemia. The lack of hyperinsulinemia suggests that there was impaired β-cell function in the obese Ossabaw swine, which could be caused by a number of obesity related complications including ectopic lipid deposition in the pancreas. Although there was no ectopic lipid deposition in the liver or longissimus dorsi muscle. Challenging Ossabaw swine with insulin revealed that high dietary palm oil induced insulin resistance, but the addition of dietary n-3 fatty acids attenuated the palm oil-induced insulin resistance. Despite the hyperglycemia and insulin resistance, the capacity to markedly expand adipose tissue may have afforded protection against obesity-linked inflammation. This demonstrates, at least in this study, that inflammation is not definitely linked to inflammation, at least in the parameters we measured.

Due to inflammation being largely absent, we hypothesized that the ability to expand adipose tissue protected Ossabaw swine against adipose tissue inflammation. Therefore, we determined whether SVC-derived adipocytes from Ossabaw swine were protected from palmitate-induced inflammation and insulin resistance compared to Yorkshire SVC-derived adipocytes (Chapter 4). We determined that NFκB was equally activated in both Ossabaw and Yorkshire adipocytes in response to palmitate. However, only Yorkshire adipocytes

secrete the chemokine IL-8 in response to palmitate treatment. IL-8 is responsible for recruiting neutrophils to the site of injury and inflammation, and are present in adipose tissue after one week of high fat feeding in mice (1). The absence of palmitate-induced IL-8 production by Ossabaw adipocytes may be involved in the lack of substantial adipose tissue inflammation seen in our first study. Consistent with the lack of palmitate-induced IL-8 production in adipocytes, there was no increase in serum or adipose tissue IL-8 in obese Ossabaw swine.

Palmitate decreased insulin-stimulated glucose uptake in both adipocyte genotypes. However, Ossabaw adipocytes had decreased basal and insulin-stimulated glucose uptake compared to Yorkshire adipocytes. Although the fold increase for insulin-stimulated glucose uptake was equal between the two adipocyte genotypes. Though there have been no direct comparisons, this *in vitro* data matches two separate *in vivo* insulin challenges in Ossabaw and Yorkshire swine, where Yorkshire swine comparably have more insulin sensitivity than Ossabaw swine. Whether this decreases overall insulin sensitivity of Ossabaw swine contributes to the susceptibility of Ossabaw swine to develop insulin resistance remains to be determined.

Because of the potential for adipose expansion to protect against inflammation, we performed microarray analysis on subcutaneous and visceral adipose tissue of lean and obese Ossabaw swine (Chapter 5). Therefore, Ossabaw swine were fed either a restricted or an *ad libitum* nutritionally adequate diet for 38 weeks. Microarray analysis revealed no dietary treatment induced differences in gene expression after controlling for FDR. Coinciding with this, there were no negative obesity-linked metabolic alterations. The lack of a high fat diet is likely causative to the lack of metabolic alteration in obese Ossabaw swine. The

importance of high fat in inducing metabolic alterations is evident in the fructose group of Lee *et al.* (7) which did not cause a negative metabolic profile. There were 1494 differently expression genes by depot after controlling for FDR, of which 777 were annotated. One gene ontology cellular component and two KEGG pathways were determined to contain over-represented genes from our significantly expressed genes by depot. These were extracellular region, arachidonic acid metabolism, and chemokine signaling pathway, respectively.

Overall, we have demonstrated that Ossabaw swine have a favorable inflammatory profile in response to a high fat diet, despite morbid obesity. Additionally, the inflammatory profile was not linked to negative metabolic consequences. Potentially, the ability to expand adipose tissue protects against inflammation, which is supported by palmitate induced IL-8 induction in Yorkshire, but not Ossabaw adipocytes. This is further evidenced by the lack of obesity-linked microarray gene expression differences in Ossabaw swine when fed a diet without high fat.

This research can be furthered in multiple areas. Perhaps most intriguing is whether the decreased basal and insulin-stimulated glucose uptake in Ossabaw adipocytes versus Yorkshire adipocytes is a component of the ability to Ossabaw swine to develop insulin resistance. Additionally, genetic determination of the cause of the reduced glucose uptake and whether analogous conditions is a component of obesity-linked insulin resistance in humans. Additionally, continued research into the capacity of Ossabaw swine to expand adipose tissue and its ability to afford protection against obesity-linked inflammation is an intriguing question, particularly if it is analogous to the metabolically healthy obese human population subset. Finally, because of immunological differences between humans and mice,

further characterization of swine adipose tissue macrophages with regards to phenotype and function is an imminent research question to further the validation of the model for obesity and obesity-linked research.

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